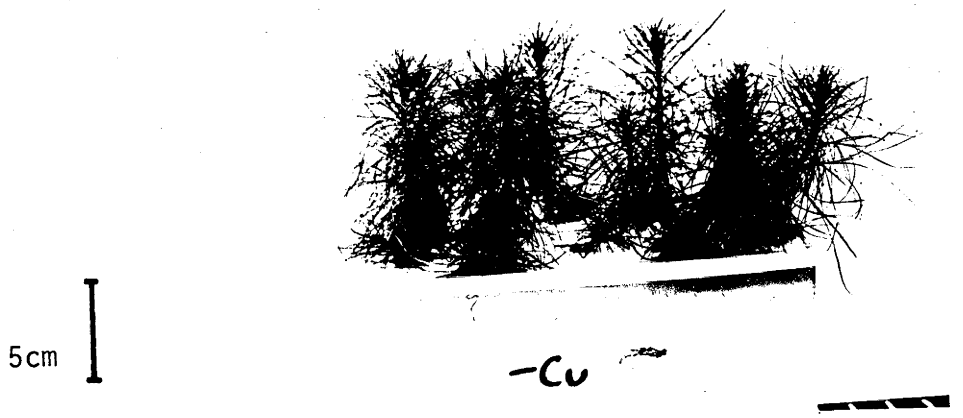
(a)



(b)

(c)

Front piece : 24 week old seedlings of Pinus radiata D.Don in nutrient solutions which are (a) Zn deficient, (b) Cu deficient, and (c) full nutrient.

STUDIES ON HEAVY METAL NUTRITION OF *PINUS RADIATA* D.DON

by

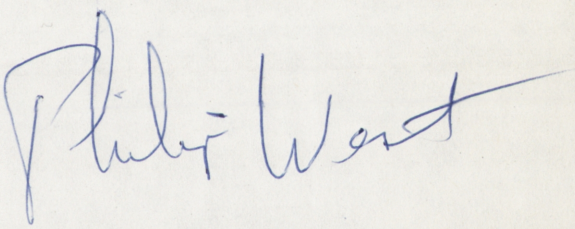
Philip Wallace West

A thesis presented for the degree of Doctor of Philosophy of the Australian National University, Canberra.

February 1976

DECLARATION OF ORIGINALITY

Except where specifically acknowledged in the text, this work is entirely that of the author.



Philip W. West

February 1976

ACKNOWLEDGEMENTS

I was supported throughout the duration of this work by a Commonwealth Postgraduate Research Award.

I am indebted to Dr E.P. Bachelard, whose guidance, assistance and counsel throughout this project were invaluable.

This thesis was printed on a Hewlett-Packard HP2100S computer using Mr G.R. Archer's Document Printing Programme.

TABLE OF CONTENTS

PAPER 1 (Continued)

PAPER 2 (Continued)

PAPER 3 (Continued)

PAPER 4 (Continued)

CONCLUSION (Continued)

LIST OF TABLES

PAPER 3 (Continued)

PAPER 4

PAPER 5

LIST OF FIGURES

PAPER 3 (Continued)

PAPER 4

PAPER 5

STUDIES ON HEAVY METAL NUTRITION OF *PINUS RADIATA* D.DON

ABSTRACT

A study was made of the factors controlling the Cu, Zn, Mn and Fe nutrition of seedlings of Pinus radiata D.Don grown for up to three months in solution culture. The effects of Cu and Zn deficiency on growth and some metabolic processes were also considered.

Levels at which Cu and Zn became insufficient for maximum growth both in solution culture and internally within roots and shoots were estimated. Mathematical relations between growth and seedling nutrient concentration were determined. A study of pH effects showed that seedling growth was progressively reduced by changing the pH of the nutrient solution from 4-7. P. radiata seemed more pH sensitive than some other species.

Heavy metals accumulated in roots when present in the external solution at above sufficiency levels. The protein extracted from roots with water and hot tri-chloro acetic acid appeared to store this excess metal. When plants with excess metal in roots were no longer supplied with sufficient metal for uninterrupted growth, the excess metal was released to shoots. In conjunction with this work, studies were made of methods to extract and assay protein and nucleic acids from P. radiata tissue.

The effects of Cu and Zn deficiencies on amino

acid, protein, sugar, phosphorus, nitrogen and photosynthetic pigment levels in seedlings were examined briefly and related to the known metabolic roles of these substances in plants.

Reports that heavy metals compete for uptake by roots were not supported here. Other work has often used abnormally high levels of competing ions; this was not the case in this study. Current theories, based mainly on work with alkali cations, propose there are dual, active ion-uptake mechanisms which operate in the cell at different external ion concentrations. There was little evidence here that similar mechanisms operate with heavy metals. The mechanisms for uptake of these metals by roots, although different for each metal, appeared to be related. Similar, related mechanisms released metals from roots to the xylem stream. Rate of transport of metals to shoots was independent of rate of transpiration: this was discussed in relation to possible mechanisms for metal transport in the xylem. The long term nature of the experiments made interpretation of aspects of these results difficult.

The control of heavy metal uptake and transport by plant hormones was studied. A mechanism for auxin control of Zn uptake by roots was proposed. Effects on Cu and Mn transport to shoots were also discussed.

INTRODUCTION

THE AIMS, SCOPE AND PRESENTATION OF THIS STUDY

STIMULUS FOR THE TOPIC - HEAVY METAL NUTRITION

Deficiencies of heavy metals have seriously affected growth of Pinus radiata D.Don in Australian forests. In particular, Zn deficiency has been observed on sands in Western Australia (Smith and Bayliss 1942) and in South Australia where two-year-old plantations are now routinely sprayed with zinc sulphate to avoid the deficiency. The stag-headed trees that result from Zn deficiency are quite unsuitable for wood production. Cu deficiency has been observed in three-year-old P. radiata growing on highly leached sands in South Australia (Ruiter 1969) and in pot trials with sands from Victoria (Hall 1961). Ruiter observed very marked distortion and die-back of leading shoots, while the symptoms observed by Hall in younger seedlings were not as severe.

From a purely practical point of view then, the heavy metal nutrition of P. radiata is a worthy subject of study, given the importance of this species to Australian forestry.

THE APPROACH OF THIS WORK TO HEAVY METAL NUTRITION

This work aimed to examine the mechanisms within

the plant that control the uptake, transport and use of heavy metals by the plant over longer time periods (i.e. several months). It was not thought of as a field study to investigate specific field problems with heavy metal nutrition. Nor was it designed as a laboratory study, the results of which might be directly applicable to some field situation. Rather through the more fundamental approach adopted here, it was hoped to expand knowledge of the overall processes of plant nutrition. The results may also, though not necessarily, have had some field application.

The general philosophy of this work might be summed up as an attempt to examine the control of plant nutrition processes *per se*, using *P. radiata* as the test species and the heavy metals Cu, Zn, Mn and Fe, with emphasis on the first two, as the test ions.

In practical terms, it is only feasible in such a study to use seedlings. Where close control of the availability of nutrients to the plant is required, solution cultures are essential but for the most part impractical with large trees, although such studies have been made (Hewitt 1966). In terms of the time scale of growth of large trees, it is also only possible in a three year study such as this to use young seedlings. An integrated study of the nutrition of *P. radiata*, as a particular species, would hardly be complete if only the first few months of the life of the tree were studied. But given the objects of this work, study of seedling nutrition is just as relevant as that of full grown

trees.

AREAS STUDIED AND DEVELOPMENT OF THE PROJECT

At the outset of this project, two aspects of heavy metal nutrition seemed obviously in need of study. Firstly, to follow the behaviour of any species under metal-sufficient and -deficient conditions, it is necessary to know at what plant nutrient levels that species becomes deficient. Data relating to Zn and Cu deficiency in P. radiata seemed to be lacking. Secondly, it seems to be generally accepted in much of the literature that heavy metals compete for uptake at the root surface: this seems feasible, considering the proximity of these elements to each other in the periodic table. Studies of this phenomenon in seedlings of P. radiata were also lacking. The first of the five papers that follow, detailing the experimental work of this study, reports findings on both these questions. Further studies on the competition for uptake between metals are reported in the second paper.

The results from these studies established sufficiency levels, for uninterrupted growth of P. radiata, both in the nutrient solution and internally in roots and shoots for both Cu and Zn. Little evidence was found to suggest there was competition for uptake between these metals. These latter results are discussed in relation to current theories of the ion uptake mechanisms of plants and differences between the conditions of this and other reported studies.

One fact that emerged from these first experiments was that very high levels of Cu, Zn, Mn and Fe accumulated in roots when these elements were present in the nutrient solution at above sufficiency levels: the effect became much exaggerated as the nutrient level rose. This finding was further pursued as it appeared to demonstrate either a defence mechanism of the plant, whereby excess metal from solution was stored to prevent toxic levels accumulating in the plant, or a safety mechanism, whereby the plant stored excess metal against times of nutrient deficiency. Attempts were made to determine the chemical fractions of the roots and shoots with which these metals were associated, in what root tissues the excess metal was stored, and whether or not the stored metal was released to shoots when the seedling received a sub-sufficient metal supply. Substantial results, reported in the second and fourth papers, were obtained which gave information on these questions and suggested some further avenues for research. In pursuing this work, problems were encountered with the extraction and assay of protein and nucleic acids from P. radiata tissue. The third paper presents a largely technical report of these problems and the techniques developed to overcome them.

As a secondary aim in the work reported in both the fourth and fifth papers, data were accumulated which gave information on the metal uptake and transport mechanisms of the plant.

At this stage, towards the end of the time

available for this work, one aspect which seemed overlooked was study of the factors which integrate the mechanisms by which heavy metals are absorbed by plants and distributed to the various meristems. A completely new line of research was adopted and preliminary evidence for control of plant nutrition by plant hormones was obtained. These results are discussed in the fifth paper. A hypothesis is proposed for control of Zn nutrition by auxin; effects on Cu and Mn nutrition by plant hormones were also observed.

PRESENTATION OF RESULTS AND CONCLUSIONS

As discussed above, the results of the experimental work here are presented as five complete and separate papers. Each was deliberately designed to be quite independent of the others except where they overlapped in experimental techniques or the work of a later paper was directly stimulated by the results of an earlier one. The results of each paper are generally discussed within the restricted frame of reference of that paper, not necessarily agreeing with the results of earlier papers and not necessarily expanded to the broader areas of the topic. The papers were written, and are presented, in chronological order. Some experimental work which was not successful, nor worthy of presentation as a complete paper, is not discussed till the concluding paper.

The work concludes with a paper which aims not merely to summarise all the details of the experimental work; these are discussed in the individual papers. Rather, it

attempts to present an integrated view of the present theories concerning the control of plant nutrition by plant processes and to relate the results obtained in this work to these theories where appropriate.

PAPER 1

GROWTH AND HEAVY METAL NUTRITION OF SEEDLINGS OF *PINUS RADIATA* D.DON WITH PARTICULAR REFERENCE TO ZINC AND COPPER

ABSTRACT

Seedlings of *P. radiata* were grown in solution culture for about 20 weeks. In one experiment Zn or Cu was varied from deficient to sufficient levels; in a second, pH, Zn and Cu levels were varied. Seedling weights, N, P, Cu, Zn, Mn and Fe levels in shoots and roots and photosynthetic pigments in shoots were assayed. The relation between weight of shoot or root and Cu or Zn concentration in shoot or root was part of a sigmoid curve. Root growth required higher Cu levels than shoot growth, while Zn requirements for both were similar when metal concentrations were expressed relative to tissue water content. Little competition between metals for uptake by roots was observed contrary to other results reported in the literature. When in adequate supply the metals accumulated in the root, suggesting this was a storage site for excess metal. Increasing the pH of the nutrient solution from 4-7 reduced growth, uptake of metals and $HPO_4^=$ and levels of photosynthetic pigments.

INTRODUCTION

Zn and Cu are essential micronutrients required for growth of most plants. Much work has been done to determine levels of Zn and Cu necessary for unimpaired plant growth (Hewitt 1966, Thorne 1957) but knowledge of specific requirements for root and shoot growth of P. radiata is lacking.

Shoot growth relies on supply of nutrients by the roots, so factors affecting uptake of metals by roots may be important in determining subsequent supply to shoots. In this regard, changes in H^+ concentration in the nutrient medium may be important (Robertson 1958). The concentration of other ions may also affect uptake of a particular ion: Zn and Cu have been shown to compete for root uptake both with each other and with other metals (Smith 1962).

The experiments described here were designed to examine the effects of changing Zn and Cu supply on growth and nutrient status of roots and shoots of P. radiata seedlings. Some factors affecting uptake of Cu, Zn, Mn and Fe by roots were examined and supply of metals to shoots by roots was discussed. Attempts were made to relate the results to the known physiology of these metals in plants.

MATERIALS AND METHODS

Experimental

Effects of Zn and Cu levels

P. radiata seeds from one poly-crossed mother clone were sown in coarse sand and watered from the tap. Eight weeks after sowing, seedlings were transferred to 5l plastic pots. Twenty seedlings per pot were supported with foam rubber in holes in the lid.

Nutrient solutions were not aerated. They contained: $Ca(NO_3)_2$ 1.05mM, $MgSO_4$ 1.02mM, K_2HPO_4 0.51mM, FeEDTA 86μM, H_3BO_3 46μM, $MnCl_2$ 9.1μM, $Co(NO_3)_2$ 0.17μM, $NaMoO_4$ 0.10μM. All macronutrients were freed of traces of Zn and Cu by co-precipitation with $Mg(OH)_2$ (Munns and Johnson 1960). Solutions were changed weekly. pH was initially adjusted to pH 4 with dilute HCl. Glass distilled water was used throughout. Seedlings were grown in a glasshouse with an overall temperature range 20-35°C. Additional light was provided with fluorescent tubes to give 18hr days.

The experiment was divided into a Zn and Cu section. In the Zn section all pots received 0.02ppm Cu (as $CuCl_2$) and Zn (as $ZnSO_4$) at 0, 0.005, 0.01, 0.05, 0.1, 0.5 or 1.0ppm. In the Cu section, all pots received 0.05ppm Zn and Cu at 0, 0.002, 0.005, 0.01, 0.05, 0.1 or 1.0ppm. The experiment was replicated twice giving 28 pots in all. The plastic pots did not exclude all light and some algal growth occurred in the solutions. Seedlings were harvested at 8, 12 and 19.5 weeks from the time of application of the treat-

ments. Roots were carefully washed with deionised water to remove algal growth before weighing.

Interaction of pH, Zn and Cu

Seedlings were sown and transferred to nutrient solutions as described above. Three treatments were applied as a 4×2^2 factorial with two replicates in randomized blocks. The first factor was pH which was adjusted at the weekly change of nutrient solutions and half-way through each week. Levels were pH 4,5,6 and 7. Between adjustments pH rose steadily (Fig 1). The results, therefore, represent the effects of pH meaned over the pH change between adjustments. Clearly these means follow the same trend as the initial pH levels (Fig 1), although at lower pH levels the tendency to rise was much greater. The second and third factors were Zn and Cu respectively at 0.05 and 0.4ppm for Zn and 0.007 and 0.07ppm for Cu. These were chosen as marginally sufficient and quite adequate levels of these elements so that, if the elements competed for uptake, the higher level of one might induce deficiency symptoms of the other even when the other was in just adequate supply.

Seedlings were harvested at 15 and 23 weeks after the treatments were applied.

Measurements

Seedlings were divided into shoots and roots,

weighed fresh, then dried overnight in a forced draught oven at 85°C and reweighed.

Total N and P in dry samples were determined from all but the first harvest of the first experiment. The colorimetric methods of Fogg and Wilkinson (1958) for P and Anon (1971) for N were used after digestion in concentrated H_2SO_4 with 200g/l K_2SO_4 and 1g/l Se (Jackson 1958). Total Cu, Zn, Mn and Fe were determined with an atomic absorption spectrophotometer after digestion in 1:7:24, H_2SO_4:$HClO_4$:HNO_3.

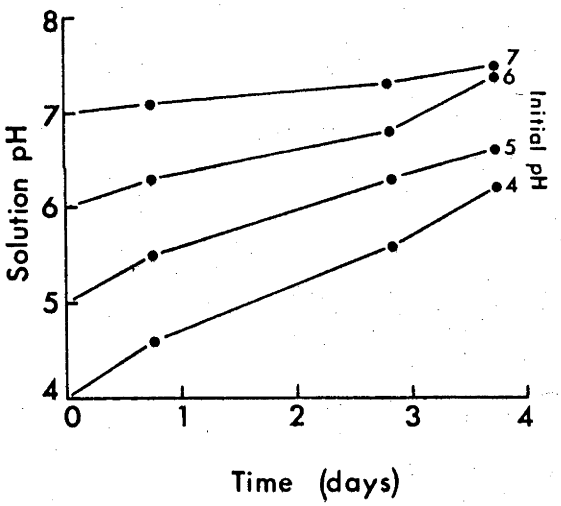


Fig 1 Change in pH of nutrient solutions over a four day period. Results were averaged over three pots and measured in the twelfth week after treatments were applied.

In the last two harvests of the first experiment and the first harvest of the second, photosynthetic pigments from the top 1cm of shoots were determined after extraction in 80% acetone (Oelke and Andrew 1966). Chlorophylls a and b were estimated from the formulae of Arnon (1949) and carotene

from that of Jaspars (1965).

Analysis of results

Results from the first experiment were treated as a 7 x 3 x 2 factorial for variance analysis. The factors were Zn or Cu level, time of harvest and replicates. The second experiment was analyzed as a 4 x 2^4 factorial, the factors being pH, Zn level, Cu level, time of harvest and replicates. Data for shoots and roots were analyzed separately in each experiment. Some missing values occurred in the data due to accidental loss of material. These were estimated by the method of Cochran and Cox (1957) and one degree of freedom was subtracted from the within groups degrees of freedom for each missing value.

Data such as those collected here are commonly heteroscedastic when untransformed. Tables 1-3 show the results of Bartlett's test (Sokal and Rohlf 1969) for homoscedasticity for several arbitrarily selected parameters measured in the second experiment, both with and without logarithmic transformation. Results for all other parameters measured in both experiments were similar to those shown in the Tables. For the untransformed data, variances commonly increased as means increased, particularly where differences between means were very large. Sometimes variances were homogeneous and at other times not. But when the data were transformed to logarithms, variances became homogeneous whether they were homogeneous or not in the untransformed

Table 1 Results of Bartlett's test for homogeneity of variance for selected parameters from the second experiment both with and without logarithmic transformations. The results show the main effect of pH. Means (x) and variances (S^2) for each parameter are shown, together with the level of significance (p<) of the tests for heteroscedasticity. Oven dry weight (ODW) measured in g, metal concentrations (Conc) in ppm relative to ODW, amounts (Wt) of metals as µg metal per seedling. Sts = Shoots, Rts = Roots.

		Untransformed					Logarithmic transformation				
pH		4	5	6	7	p<	4	5	6	7	p<
ODW	x	1.6	1.3	1.0	0.7		-.28	-.14	-.07	-.45	
Sts	S^2	.67	.36	.23	.08	.005	.38	.23	.22	.15	NS
ODW	x	.39	.37	.29	.25		-1.0	-1.1	-1.3	-1.5	
Rts	S^2	.03	.02	.01	.01	NS	.18	.12	.15	.32	NS
Conc Zn	x	32.	26.	21.	18.		3.3	3.1	2.9	2.7	
Sts	S^2 *	3.3	1.8	1.5	1.0	NS	.30	.28	.49	.39	NS
Wt Zn	x	42.	31.	21.	12.		3.6	3.3	2.8	2.3	
Sts	S^2 *	5.3	3.3	2.5	.7	.001	.29	.32	.55	.52	NS
Conc Zn	x	63.	59.	40.	30.		3.9	4.0	3.6	3.3	
Rts	S^2 *	18.	9.	3.	2.	.005	.41	.30	.18	.26	NS
Wt Zn	x	21.	21.	13.	7.		2.9	2.9	2.3	1.8	
Rts	S^2 *	.9	1.6	.7	.2	.005	.24	.41	.49	.32	NS
Conc Mn	x *	1.3	1.4	1.8	1.1		4.8	4.8	5.1	4.5	
Sts	S^2 *	20.	28.	56.	38.	NS	.13	.17	.26	.29	NS
Wt Mn	x *	1.8	1.5	1.7	0.7		5.1	5.0	5.0	4.1	
Sts	S^2 *	28.	20.	83.	12.	.005	.11	.08	.22	.23	NS
Conc Mn	x *	2.5	4.6	6.4	5.2		5.5	6.1	6.4	6.2	
Rts	S^2 *	70.	336.	536.	227.	.01	.09	.17	.17	.08	NS
Wt Mn	x *	1.0	1.7	1.8	1.3		4.5	5.0	5.1	4.7	
Rts	S^2 *	61.	73.	72.	59.	NS	.32	.26	.21	.41	NS

* Multiply figures in untransformed columns by 100

Table 2 Data similar to those of Table 1, but for the main effects of Zn and Cu treatments

	Zn treatments				Cu treatments			
	Untransformed			Logarithmic			Untransformed			Logarithmic		
	.05 ppm	.4 ppm	p<	.05 ppm	.4 ppm	p<	.007 ppm	.07 ppm	p<	.007 ppm	.07 ppm	p<
ODW x Sts S^2	1.1	1.2		-.08	-.03		1.0	1.2		-.10	-.05	
	.45	.40	NS	.32	.30	NS	.30	.54	NS	.28	.33	NS
ODW x Rts S^2	.33	.31		-1.2	-1.3		.30	.34		-1.3	-1.2	
	.02	.02	NS	.20	.25	NS	.02	.02	NS	.27	.18	NS
Conc Zn x Sts S^2 *	14.	34.		2.5	3.5		27.	21.		3.1	2.9	
	0.4	1.7	.005	.24	.12	NS	2.8	1.2	.025	.40	.39	NS
Wt Zn x Sts S^2 *	15.	38.		2.5	3.5		27.	25.		3.0	3.0	
	1.3	4.1	.005	.50	.25	NS	5.1	3.0	NS	.63	.68	NS
Conc Zn x Rts S^2 *	29.	67.		3.3	4.1		53.	43.		3.8	3.6	
	2.	11.	.001	.17	.21	NS	14.	6.	.025	.40	.30	NS
Wt Zn x Rts S^2 *	11.	20.		2.1	2.8		15.	15.		2.5	2.5	
	0.8	1.2	NS	.53	.38	NS	1.2	1.2	NS	.60	.58	NS

Table 2 continued overleaf.....

Table 2 (continued)

	Zn treatments						Cu treatments					
	Untransformed			Logarithmic			Untransformed			Logarithmic		
	.05 ppm	.4 ppm	p<	.05 ppm	.4 ppm	p<	.007 ppm	.07 ppm	p<	.007 ppm	.07 ppm	p<
Conc Mn x *	1.4	1.4		4.8	4.9		1.4	1.4		4.9	4.8	
Sts S^2 *	44.	38.	NS	.24	.23	NS	32.	50.	NS	.12	.28	NS
Wt Mn x *	1.3	1.5		4.7	4.9		1.3	1.5		4.8	4.8	
Sts S^2 *	68.	40.	NS	.38	.28	NS	34.	74.	.05	.27	.40	NS
Conc Mn x *	4.9	4.4		6.1	6.0		4.9	4.5		6.1	6.0	
Rts S^2 **	56.	40.	NS	.24	.23	NS	62.	34.	NS	.26	.22	NS
Wt Mn x *	1.5	1.3		4.9	4.7		1.4	1.5		4.8	4.8	
Rts S^2 *	85.	59.	NS	.31	.38	NS	66.	81.	NS	.40	.30	NS

* Multiply figures in untransformed columns by 100 or
** by 1.000

Table 3 Data similar to those of Tables 1 & 2, but for the main effect of time of harvest (i.e. 15 or 23 weeks from the time of establishment of the experiment).

	Untransformed			Logarithmic		
	Harvest (wks)			Harvest (wks)		
	15	23	p<	15	23	p<
ODW $\bar{x}$	0.7	1.6		-.45	.39	
Sts S^2	.04	.37	.005	.09	.17	NS
ODW $\bar{x}$	.25	.39		-1.5	-1.0	
Rts S^2	.01	.02	NS	.21	.13	NS
Conc Zn $\bar{x}$	29.	19.		3.3	2.8	
Sts S^2 *	2.3	1.3	NS	.26	.42	NS
Wt Zn $\bar{x}$	20.	33.		2.8	3.2	
Sts S^2 *	1.4	5.9	.005	.40	.86	.05
Conc Zn $\bar{x}$	51.	45.		3.8	3.6	
Rts S^2 *	12.	7.	NS	.38	.32	NS
Wt Zn $\bar{x}$	13.	18.		2.3	2.7	
Rts S^2 *	0.9	1.4	NS	.63	.47	NS
Conc Mn $\bar{x}$ *	1.8	1.0		5.1	4.5	
Sts S^2 *	29.	20.	NS	.11	.16	NS
Wt Mn $\bar{x}$ *	1.2	1.6		4.7	4.9	
Sts S^2 *	20.	81.	.005	.23	.42	NS
Conc Mn $\bar{x}$ *	4.9	4.5		6.1	6.0	
Rts S^2 **	42.	54.	NS	.20	.27	NS
Wt Mn $\bar{x}$ *	1.2	1.7		4.6	5.0	
Rts S^2 **	3.	10.	.005	.29	.35	NS

* Multiply figures in untransformed columns by 100 or ** by 1000

data. Further tests showed that the error variates in each sample were normally distributed after the logarithmic transformation, whereas they were sometimes skewed before transformation. The logarithmic transformation was used for all the analyses of variance of data from both experiments.

The results of these analyses (Tables 4-7) are reported as means back-transformed from the logarithmic means. Where several means were to be compared after a significant result in variance analysis, the least significant difference test (i.e. a t test) was used (Sokal and Rohlf 1969). This procedure is theoretically inaccurate when more than two means are being tested and a multiple range test is often preferred. But the simpler t test showed the trends in the results from the experiments quite satisfactorily. Because the means reported in Tables 4-7 were back-transformed from logarithms, the actual values of the least significant differences are not shown, for these too were calculated as logarithms and their back-transformations are not directly comparable with the back-transformed means. Instead, means which did not differ significantly (all tests were done with a 95% confidence limit) are superscripted with the same letter. In variance analyses F tests significant with a probability greater than 95% were considered to represent real effects of treatments.

RESULTS

Deficiency symptoms

About one month after initial application of treatments, seedlings receiving very low levels of Zn produced short, thick needles at the growing tip giving a flat-topped appearance. Needles when fully developed were only 1-2cm long compared with 3-5cm in normal plants. Roots showed no gross morphological effects of Zn deficiency.

Cu deficiency did not produce marked symptoms apart from an obvious reduction in growth rate about 6-8 weeks after treatments were applied.

Both Zn and Cu deficient seedlings are shown in the Frontpiece to this thesis.

Effects of Zn, Cu and pH on growth

Significant effects of Zn and Cu levels of the nutrient solution on shoot growth were observed only at the third harvest of the first experiment. Maximum shoot growth occurred with about 0.05ppm or more Cu (Table 4) or Zn (Table 5) in the external solution. Similar trends were observed in root growth at this harvest but the differences were not large enough to be significant (Tables 4,5).

In the second experiment, Zn was supplied at just adequate (0.05ppm) or above adequate (0.4ppm) levels and so Zn level did not significantly affect root or shoot growth

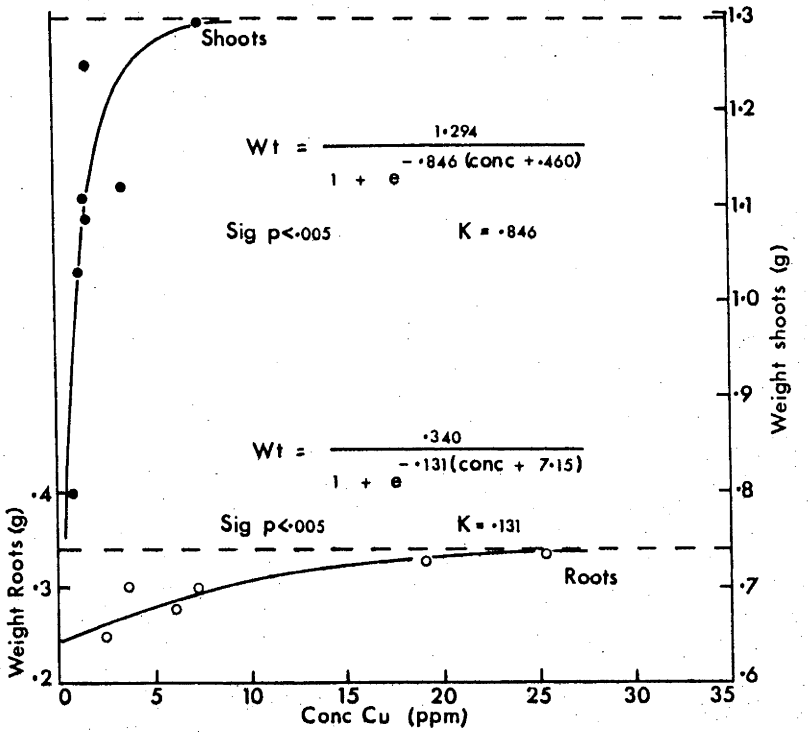
even by the time of the second harvest (Table 7). Cu was supplied at below adequate (0.007ppm) or above adequate (0.07ppm) levels: by the time of the second harvest, shoot growth was reduced with inadequate Cu but the effect on root growth was not large enough to be significant (Table 7).

To determine if roots and shoots differed in their growth response to metal deficiency (because of differing physiological demands), mathematical models were developed, from the data of the first experiment, to relate growth to internal plant nutrient concentration.

Plots of growth against dry weight concentration of Zn or Cu at the third harvest (Figs 2,3) suggested that the relation in both roots and shoots was at least part of a sigmoid curve. Ricklefs (1967) discusses a method to determine if data approximate to one of three possible sigmoid curves, the logistic, Gompertz or von Bertalanffy curves which all differ somewhat in shape. This method determines the equation of the tangent to the curve at its inflection point: the slope of this line is, by convention, taken as a rate constant for the curve. Statistics to determine the equation of the curve are calculated at the same time. Using this method, it was apparent that the data here could best be explained by the logistic curve. The equation of this curve is given by:

$$y = 1/(1 + be^{-kx})$$

where y is a dependent and x an independent variable and b and k are constants. Fig 4 shows a typical curve of this



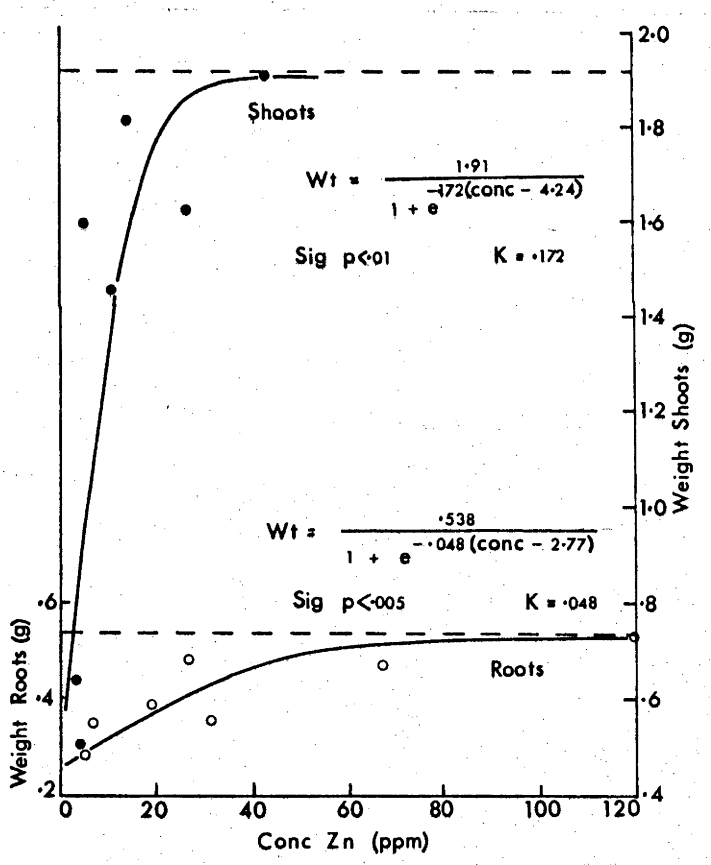


Fig 2 (Upper) and Fig 3 (Lower). Weight (g per seedling) of shoots and roots of seedlings from the first experiment at 19.5 weeks from the time of application of the treatments. Weight is expressed as a function of internal shoot or root Cu (Fig 2) or Zn (Fig 3) concentration (ppm relative to oven dry weight). Equations of the curves, significance levels of the equations and rate constants of the curves are also shown.

type.

The equation of the least-squares curves fitted to the data of Figs 2 and 3 by Ricklef's method are shown on the Figures. The values of the rate constant, K, and the level of statistical significance of the equations are also shown. Clearly, the rate constants for the curves for shoots far exceeded those for roots. The inflection points for all these curves occurred slightly to the left of the Y-axis. The Cu concentration, relative to dry weight, for maximum shoot growth was about 6ppm and for roots about 20ppm. Maximum shoot growth occured with 30ppm Zn and root growth with 70ppm.

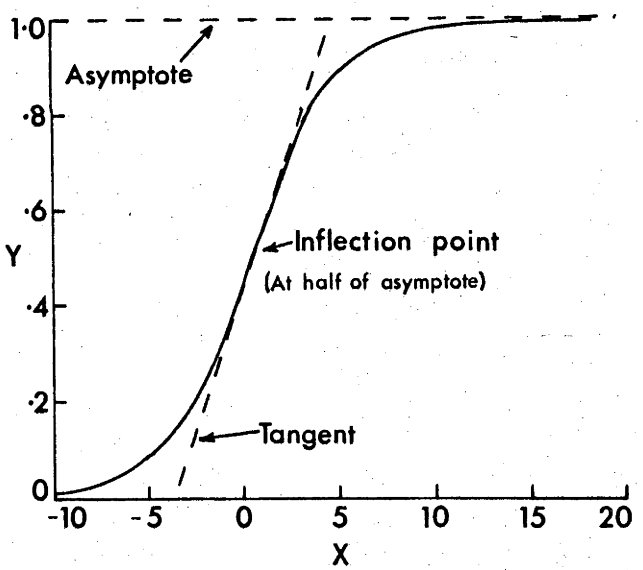


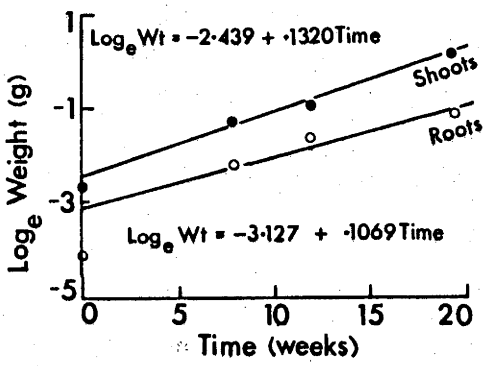
Fig 4 A typical logistic sigmoid curve. For this curve:

$$y = 1/(1 + e^{-.5x}) \qquad K = .5$$

The curve approaches the asymptote shown as x becomes large. The tangent to the curve at the inflection point is also shown.

In physiological terms, these models seem unsatisfactory for two reasons. Firstly, when absolute values of root and shoot growth were plotted against time, it was found that over any time period absolute growth rate of roots was less than absolute growth rate of shoots. Over any time period then, the absolute growth response of roots to a given change in nutrient concentration should be less than the absolute growth response of shoots, assuming root and shoot nutrient physiologies are similar. To compare responses of roots and shoots to different nutrient concentrations, a transformation of growth data is required to reduce shoot and root growth rates to comparable bases. Figs 5 and 6 show the growth curves with time of shoots and roots in both sections of the first experiment meaned over all Zn or Cu treatments (i.e. the main effect of time in variance analysis) with growth expressed as the logarithm of absolute growth. The data for zero time were derived from only one measurement which may explain their apparent deviation from the straight lines. The regression equations shown were derived after a test of the null hypothesis that the slopes and intercepts of the equations did not differ significantly. In the Cu section of the experiment, the best model explaining these results was significant with $p<.001$, explained 92% of the variance in the data and showed the slopes and intercepts of both regression equations differed significantly ($p<.025$). That is relative growth rate of roots was slightly below that of shoots; but the difference was probably not large enough to affect the conclusions that

follow. In the Zn section the best model to explain the data was significant with $p<.001$, explained 92% of the variance and showed the intercepts of the two lines differed significantly ($p<.001$) but not the slopes. That is, relative growth rates of roots and shoots were similar. These results suggest that it is desirable to use relative (logarithmic) growth rates rather than absolute growth rates to ensure that any differences between root and shooot growth are differences in response to nutrients not differences in absolute growth rate.



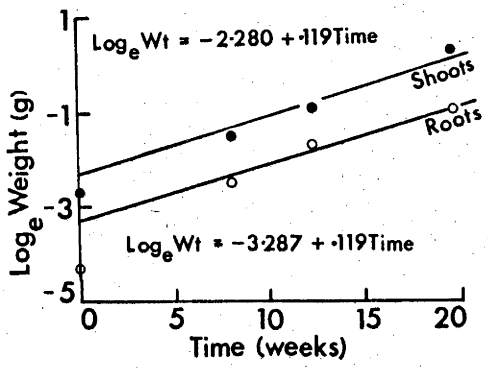


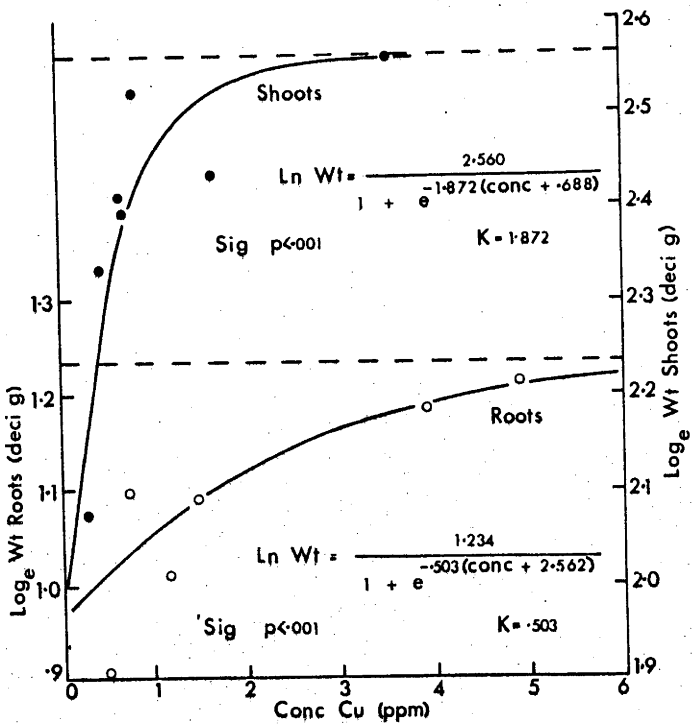
Fig 5 (Left) and Fig 6 (right). Growth ($\log_e$ oven dry weight per seedling (g)) as a function of time in the Cu section (Fig 5) and Zn section (Fig 6) of the first experiment. Results are meaned over all treatments (i.e. the main effect of time is shown). The regression equations of the least squares lines of best fit are also shown.

The second objection to the growth models (Figs 2,3) is that concentrations relative to dry weight may bear little relation to the concentration of elements in the cytoplasm which determines their biochemical activity.

New growth models were estimated using $\log_e$ dry weight and plant internal concentration of Cu or Zn relative

to water content of shoots or roots (Figs 7,8). For the Cu section (Fig 7), the model which best explained the straight lines obtained by Rickleff's conversions showed the lines for shoots and roots had similar intercepts and different slopes. This model was significant with $p<.001$ and differed significantly ($p<.005$ at least) from other models, except the model with different slopes and intercepts. Since the model with the same intercepts but different slopes is the simpler, it was accepted as the better model. This model explained 87% of the variation in the data. The rate constant for shoots exceeded that for roots 3.7 times: the Cu sufficiency level for shoots occured at about 2-2.5ppm and for roots at 5-6ppm. This suggests that root requirements for Cu may exceed shoot requirements.

For Zn, (Fig 8), the model with different slopes and intercepts explained 77% of the variance and was significant with $p<.005$. Models with slopes only, or intercepts only, different explained 77% and 75% of the variance respectively and did not differ significantly from the model with both slopes and intercepts different. All these models explained significantly more of the variance than the model with pooled slopes and intercepts but only with $p<.10$. This last model explained 69% of the variance and was significant with $p<.001$. Clearly there were no major differences between the four models and so the model with pooled slopes and intercepts was accepted as the simplest and most appropriate. Fig 8 shows this model for roots and shoots together with the curves assuming different slopes and intercepts to emphasise



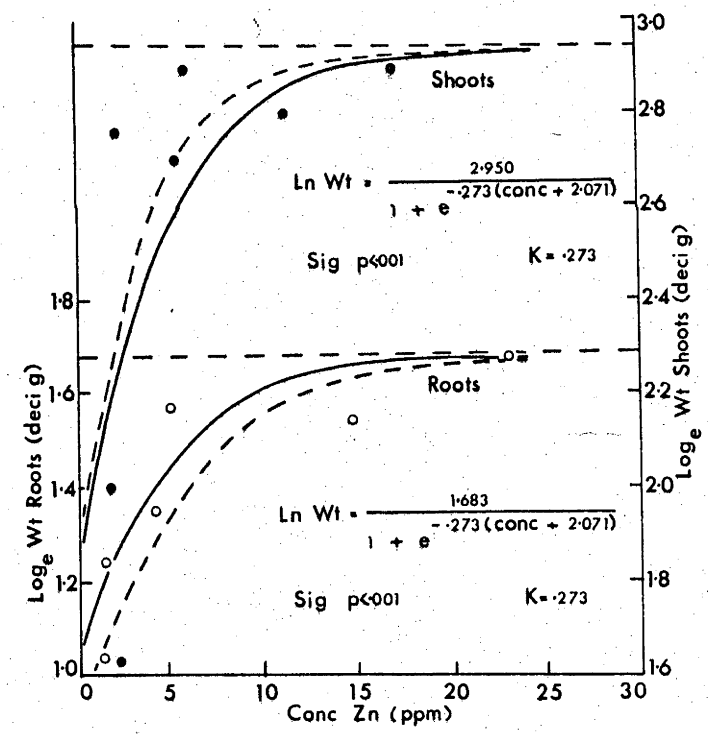


Fig 7 (Upper) and Fig 8 (Lower). Log_e weight (g per seedling) of shoots and roots of seedlings from the first experiment at 19.5 weeks from the time of application of the treatments. Weight is expressed as a function of internal shoot or root Cu (Fig 2) or Zn (Fig 3) concentration (ppm relative to water content of seedlings). Equations of the curves, significance levels of the equations and rate constants of the curves are also shown. Fig 8 —— Best regression

the similarities of these two models. It appears that root and shoot requirements for Zn are similar with 12-15ppm Zn, relative to water content, being sufficient for unimpaired growth of both shoots and roots.

Increased pH significantly reduced shoot and root growth, the effect increasing with time. By the time of the second harvest of the second experiment, seedlings grown in solutions above pH 5 showed much reduced growth (Table 6).

Effects on nutrient uptake by roots and transport to shoots

Interpretation and presentation of results

When growth occurs at different rates in seedlings which received different treatments, the interpretation of data for nutrient uptake by roots or transport to shoots becomes very difficult. Two parameters are involved. Firstly, the concentration of the nutrient in the seedling at the end of the measurement period measures the amount of nutrient taken up or transported per unit weight of the seedling. Secondly, the actual weight of nutrient in the seedling measures the availability of sites for uptake at the root surface or release to the xylem. The difficulty lies in reconciling these two parameters. Thus, the uptake or transport mechanisms of a fast growing seedling may not be able to keep pace with the growth rate: the concentration of nutrient in that seedling would then decline relative to that in a slower grown seedling. Under these circumstances, the actual amount of nutrient taken up by the faster grown seedling may

only slightly, or not at all, exceed that taken up by the slower grown seedling. Physiologically speaking, this may occur if the amount of terminal root section, which actually takes up nutrients, is the same in both fast and slow grown seedlings. This is not unlikely if tissue maturation occurs more rapidly in the faster grown seedling. In this case, the uptake rate of both seedlings would be similar, although examination of only the concentration data might suggest that the slower grown seedling had a higher uptake rate. Under conditions such as these, there is often no unequivocal answer as to which plant does show the higher uptake rate. At other times there is. Thus, if a faster grown seedling shows a higher concentration of nutrient than a slower grown, this can only mean that the former has taken up nutrient at a much faster rate than the latter.

The results obtained in this study were interpreted with these difficulties in mind. The relevant data for concentration of nutrients in roots and shoots and actual weights of nutrients transported to shoots and retained in roots of seedlings which received different treatments are shown in Tables 4 and 5 for the first experiment and Tables 6 and 7 for the second. The variance analyses suggested there was little difference in any of the effects observed at different harvests, except that often they became more pronounced at the later harvests. The data shown, therefore, are those for the final harvests in both experiments. As discussed earlier all means were back-transformed from logarithms. Probability levels at which F tests in variance

Table 4 Effects of Cu treatments on oven dry weight (ODW) and concentration (Conc) and total amounts (Wt) of nutrients and photosynthetic pigments in seedlings from the first experiment at the final harvest (19.5 weeks from establishment). Results are meaned over two replicates and are back-transformed from logarithms (see text). The significance (p<) of differences between means in variance analyses is shown. Means which did not differ significantly after a least significant difference test have similar superscripts.

ODW was measured in g, concentrations of N, P and pigments as mg/g relative ODW (N, P) and fresh weight (pigments), concentrations of Cu, Zn, Mn and Fe as ppm relative to ODW. Weights of N, P and pigments are given in mg and Cu, Zn, Mn and Fe in μg per seedling. Sts = Shoots Rts = Roots.

	Concentration of Cu in external solution (ppm)							Significance
	0	.002	.005	.01	.05	.1	1.0	p<
ODW Sts	.59	1.03	1.11^{ab}	1.09^{ab}	1.25^{ab}	1.37^{a}	1.29^{ab}	.001
ODW Rts	.36	.30	.27	.30	.33	.34	.39	NS
N Sts Conc	15.5	12.2^{a}	11.1^{a}	11.9^{a}	7.7	11.0^{a}	10.4^{a}	.005
N Sts Wt	9.2	12.5	12.2	13.7	9.5	15.4	13.4	NS
N Rts Conc	22.8	21.0	24.0	23.0	25.0	28.3	25.4	NS
N Rts Wt	8.82	6.29	6.56	6.80	8.08	8.88	9.80	NS
P Sts Conc	3.57	2.14^{a}	2.40^{a}	2.46^{a}	1.41^{b}	2.02^{ab}	2.46^{a}	.05
P Sts Wt	2.06	2.19	2.66	2.75	1.74	2.83	3.18	NS
P Rts Conc	7.41	5.74	6.00	6.10	5.96	7.15	7.35	NS
P Rts Wt	2.75	1.72	1.64	1.84	1.94	2.37	2.83	NS
Cu Sts Conc	1.2^{a}	1.1^{a}	1.5^{ab}	1.6^{b}	1.8^{b}	3.1	7.6	.005
Cu Sts Wt	0.6	1.1^{a}	1.6^{ab}	1.7^{ab}	2.2^{b}	4.1	9.8	.001
Cu Rts Conc	2.1^{a}	3.6^{a}	6.1^{b}	7.4^{b}	19.2^{c}	25.4^{c}	266.	.001
Cu Rts Wt	0.7^{a}	1.1^{ab}	1.7^{bc}	2.2^{c}	6.1^{d}	8.4^{d}	102.	.001

Table 4 continued overleaf.....

Table 4 (continued)

	Concentration of Cu In external solution (ppm)							Significance
	0	.002	.005	.01	.05	.1	1.0	p<
Zn Sts Conc	17.4	12.3[a]	11.2[a]	11.1[a]	10.0[a]	10.0[a]	12.7[a]	.05
Zn Sts Wt	11.0	12.7	12.5	11.9	12.5	13.9	16.4	NS
Zn Rts Conc	33.8[a]	21.5[ab]	19.0[b]	20.7[b]	19.2[b]	22.5[ab]	48.2	.025
Zn Rts Wt	11.6[a]	6.4[b]	5.2[b]	6.4[b]	6.2[b]	7.6[ab]	18.5	.10
Mn Sts Conc	224.	111.[a]	82.[a]	104.[a]	91.[a]	106.[a]	85.[a]	.001
Mn Sts Wt	131.[ab]	114.[bc]	91.[c]	105.[bc]	112.[bc]	146.[a]	110.[bc]	.05
Mn Rts Conc	221.[bc]	518.[b]	558.[ab]	500.[b]	477.[b]	402.[bc]	752.[a]	.001
Mn Rts Wt	80.	155.	152.	142.	153.	137.	289.	NS
Fe Sts Conc	68.3[c]	37.4[ab]	42.6[ab]	41.0[ab]	36.3[b]	37.0[b]	23.9[b]	.001
Fe Sts Wt	40.9	37.9	47.2	41.9	45.2	49.2	30.9	NS
Fe Rts Conc X.1	640.[a]	472.[bc]	510.[abc]	593.[ab]	463.[bc]	413.[c]	490.[bc]	.001
Fe Rts Wt X.1	216.	141.	139.	178.	147.	142.	188.	NS
Chlorophyll a Conc	.907	.631	.518	.558	.663	.510	.496	NS
Chlorophyll a Wt	2.69	2.29	1.82	1.95	2.66	2.23	2.04	NS
Chlorophyll b Conc	.287	.219	.178	.214	.222	.199	.198	NS
Chlorophyll b Wt	.851	.797	.595	.755	.891	.832	.811	NS
Carotenoids Conc	.213	.168	.154	.157	.169	.140	.138	NS
Carotenoids Wt	.632	.613	.539	.557	.681	.619	.565	NS

Table 5. Effects of Zn treatments on oven dry weight (ODW) and concentration (Conc) and total amounts (Wt) of nutrients and photosynthetic pigments in seedlings from the first experiment at the final harvest (19.5 weeks from establishment). Results are meaned over two replicates and are back-transformed from logarithms (see text). The significance (p<) of differences between means in variance analyses is shown. Means which did not differ significantly after a least significant difference test have similar superscripts.

ODW was measured in g, concentrations of N, P and pigments as mg/g relative ODW (N, P) and fresh weight (pigments), concentrations of Cu, Zn, Mn and Fe as ppm relative to ODW. Weights of N, P and pigments are given in mg and Cu, Zn, Mn and Fe in μg per seedling. Sts = Shoots Rts = Roots.

	Concentration of Zn in external solution (ppm)							Significance
	0	.005	.01	.05	.1	.5	1.0	p<
ODW Sts	.51^{b}	.64^{b}	1.60^{a}	1.45^{a}	1.81^{a}	1.62^{a}	1.91	.001
ODW Rts	.29^{d}	.35^{ad}	.39^{c}	.35^{cd}	.48^{ab}	.47^{b}	.54^{a}	.001
N Sts Conc	22.6	18.8	11.0^{bc}	10.5^{c}	10.9^{bc}	14.1^{ab}	14.8^{a}	.001
N Sts Wt	11.4	12.0	17.5	15.3	19.9	23.4	28.6	NS
N Rts Conc	20.8	23.2	22.1	21.7	21.3	21.7	20.5	NS
N Rts Wt	5.9	8.0	8.8	7.5	10.4	10.0	11.0	NS
P Sts Conc	4.43^{a}	4.02^{a}	2.09^{b}	1.92^{b}	2.07^{b}	2.24^{b}	2.51^{b}	.001
P Sts Wt	2.23	2.56	3.31	2.84	3.79	3.69	4.88	NS
P Rts Conc	7.55	7.00	5.94	6.50	7.29	6.72	6.55	NS
P Rts Wt	2.17	2.42	2.37	2.24	3.59	3.12	3.54	NS
Cu Sts Conc	3.6^{a}	3.4^{a}	1.0^{c}	1.8^{bc}	1.9^{bc}	1.7^{bc}	2.1^{b}	.05
Cu Sts Wt	1.8^{bc}	2.2^{ab}	1.5^{c}	2.6^{a}	3.3	2.7^{a}	4.1	.05
Cu Rts Conc	11.0	11.0	10.1	13.0	9.9	11.0	8.7	NS
Cu Rts Wt	3.1	3.8	3.9	4.6	4.9	5.1	4.7	NS

Table 5 continued overleaf.....

Table 5 (continued)

	Concentration of Zn in external solution (ppm)							Significance
	0	.005	.01	.05	.1	.5	1.0	p<
Zn Sts Conc	4.4[a]	3.7[a]	4.2[a]	11.3[b]	14.0[b]	26.4	43.1	.001
Zn Sts Wt	2.2[a]	2.4[a]	6.9[a]	16.7[b]	25.9[b]	43.0	83.8	.001
Zn Rts Conc	4.8[a]	6.8[a]	20.0[b]	31.9[b]	27.2[b]	67.3	120.	.001
Zn Rts Wt	1.4[a]	2.4[a]	8.1[a]	11.1[b]	13.8[b]	31.1	68.4	.001
Mn Sts Conc	220.[a]	186.[a]	70.[b]	76.[b]	89.[b]	83.[b]	89.[b]	.001
Mn Sts Wt	110.	119.	111.	110.	159.	132.	173.	NS
Mn Rts Conc	386.	471.	378.	463.	376.	359.	317.	NS
Mn Rts Wt	109.	163.	151.	164.	184.	164.	174.	NS
Fe Sts Conc	78.0	49.7[a]	30.4[b]	36.2[ab]	46.3[ab]	52.6[a]	38.2[ab]	.005
Fe Sts Wt	39.0[bc]	32.0[c]	48.9[bc]	53.1[b]	81.1[a]	85.1[a]	73.7[a]	.01
Fe Rts Conc X.1	788.[a]	641.[ad]	431.[d]	550.[ad]	586.[ad]	424.[d]	242.[d]	.005
Fe Rts Wt X.1	225.	223.	170.	195.	293.	192.	138.	NS
Chlorophyll a Conc	.627	.428	.627	.577	.684	.810	.605	NS
Chlorophyll a Wt	0.97[a]	0.78[a]	3.09[dc]	2.54[d]	4.26[ce]	4.41[e]	4.04[ce]	.025
Chlorophyll b Conc	.238	.179	.213	.184	.230	.303	.224	NS
Chlorophyll b Wt	0.37[a]	0.33[a]	1.05[dc]	0.81[ad]	1.45[ce]	1.67[e]	1.51[ce]	.025
Carotenoids Conc	.199	.151	.176	.171	.198	.224	.169	NS
Carotenoids Wt	0.31[a]	0.28[a]	0.86[dc]	0.75[c]	1.22[e]	1.23[e]	1.13[ce]	.005

Table 6 Main effects of pH treatments on oven dry weight (ODW) and concentration (Conc) and total amounts (Wt) of nutrients and photosynthetic pigments in seedlings from the second experiment at the final harvest (23 weeks from establishment). Results are meaned over eight observations and are back-transformed from logarithms (see text). The significance (p<) of differences between means in variance analyses is shown. Means which did not differ significantly after a least significant difference test have similar superscripts.

ODW was measured in g, concentrations of N, P and pigments as mg/g relative ODW (N, P) and fresh weight (pigments), concentrations of Cu, Zn, Mn and Fe as ppm relative to ODW. Weights of N, P and pigments are given in mg and Cu, Zn, Mn and Fe in μg per seedling. Sts = Shoots Rts = Roots.

		pH of external solution				Significance
		4	5	6	7	p<
ODW Sts		2.26^{a}	1.76^{ad}	1.40^{d}	0.87	.001
ODW Rts		.42^{a}	.44^{a}	.34^{ad}	.31^{d}	.01
N Sts	Conc	10.9	9.9	8.9	10.9	NS
	Wt	24.7^{a}	17.4^{a}	12.5^{d}	9.5^{d}	.001
N Rts	Conc	23.9	22.8	20.8	18.2	NS
	Wt	10.0^{a}	9.9^{a}	7.0^{ad}	5.6^{d}	.005
P Sts	Conc	2.18^{ad}	2.07^{d}	1.88^{d}	2.89^{a}	.025
	Wt	4.91	3.64	2.64^{a}	2.51^{a}	.001
P Rts	Conc	6.78	5.16^{a}	4.16^{a}	4.76^{a}	.001
	Wt	2.85^{a}	2.25^{a}	1.40^{d}	1.46^{d}	.001
Cu Sts	Conc	2.2	1.3	1.4	0.5	NS
	Wt	5.0^{a}	2.2^{ad}	2.0^{d}	0.5^{d}	.025
Cu Rts	Conc	13.2^{a}	13.1^{a}	11.3^{a}	7.4	.025
	Wt	5.5^{a}	5.7^{a}	3.8^{a}	2.3	.01

Table 6 continued overleaf.....

Table 6 (continued)

	pH of external solution				Significance
	4	5	6	7	p<
Zn Sts Conc	21.9[a]	18.8[a]	12.6[d]	11.9[d]	.001
Wt	49.5	33.1	17.7	10.4	.001
Zn Rts Conc	41.0[ad]	52.4[a]	40.4[d]	24.3	.001
Wt	17.2[a]	22.9[a]	13.6	7.5	.001
Mn Sts Conc	93.[ad]	89.[ad]	120.[a]	67.[d]	.05
Wt	210.[a]	156.[a]	169.[a]	59.	.001
Mn Rts Conc	209.	421.[a]	566.[d]	470.[ad]	.001
Wt	88.	184.	190.	144.	NS
Fe Sts Conc	50.6[a]	51.2[a]	38.9	43.0	.005
Wt	114.[a]	90.[a]	55.	37.	.001
Fe Rts Conc X.1	554.	372.	237.[a]	231.[a]	.001
Wt X.1	233.[a]	162.[a]	80.[d]	71.[d]	.001
Chlorophyll a Conc	.81[ad]	.90[a]	.51[dc]	.31[c]	.025
Wt	2.63[a]	2.76[a]	1.27[d]	0.55[d]	.005
Chlorophyll b Conc	.31	.27	.17	.12	.001
Wt	.95[a]	.82[a]	.43	.21	.001
Carotenoids Conc	.25	.20	.13	.09	.001
Wt	.76[a]	.60[ad]	.33[dc]	.16[c]	.005

Table 7 Main effects of Zn and Cu treatments on oven dry weight (ODW) and concentration (Conc) and total amounts (Wt) of nutrients and photosynthetic pigments in seedlings from the second experiment at the final harvest (23 weeks from establishment). Results are meaned over 16 observations and are back-transformed from logarithms (see text). The significance (p<) of differences between means in variance analyses is shown.

ODW was measured in g, concentrations of N, P and pigments as mg/g relative ODW (N, P) and fresh weight (pigments), concentrations of Cu, Zn, Mn and Fe as ppm relative to ODW. Weights of N, P and pigments are given in mg and Cu, Zn, Mn and Fe in µg per seedling. Sts = Shoots Rts = Roots.

	Conc Zn in external solution		Significance	Conc Cu in external solution		Significance
	.05	.4	p<	.007	.07	p<
ODW Sts	1.36	1.62	NS	1.39	1.59	.05
ODW Rts	.37	.37	NS	.36	.39	NS
N Sts Conc	9.6	10.6	.05	10.7	9.5	NS
N Sts Wt	13.1	17.2	NS	14.9	15.2	NS
N Rts Conc	20.8	21.8	NS	22.8	20.0	NS
N Rts Wt	7.72	8.09	NS	8.10	7.71	NS
P Sts Conc	2.19	2.26	NS	2.48	2.00	.025
P Sts Wt	2.97	3.66	NS	3.43	3.17	NS
P Rts Conc	4.92	5.35	NS	5.55	4.74	NS
P Rts Wt	1.83	2.00	NS	1.97	1.83	NS
Cu Sts Conc	1.1	1.4	NS	0.6	2.3	.005
Cu Sts Wt	1.4	2.2	NS	0.9	3.7	.005
Cu Rts Conc	10.3	11.7	NS	5.9	20.5	.005
Cu Rts Wt	3.8	4.3	NS	2.1	7.9	.001

Table 7 continued overleaf.....

Table 7 (continued)

	Conc Zn in external solution		Signif icance	Conc Cu in external solution		Signif icance
	.05	.4	p<	.007	.07	p<
Conc Zn Sts	9.4	26.4	.001	17.8	14.0	.001
Wt	12.8	42.8	.001	24.7	22.2	NS
Conc Zn Rts	26.9	54.0	.001	38.5	37.7	NS
Wt	10.0	20.0	.001	13.7	14.6	NS
Conc Mn Sts	89.8	91.2	NS	96.8	84.5	NS
Wt	122.	148.	NS	134.	134.	NS
Conc Mn Rts	418.	366.	NS	407.	376.	NS
Wt	155.	136.	NS	145.	146.	NS
Conc Fe Sts	47.6	43.8	NS	50.4	41.3	.025
Wt	64.6	70.9	NS	70.0	65.5	NS
Conc X.1 Fe Rts	318.	334.	NS	334.	318.	NS
Wt X.1	118.	123.	NS	119.	123.	NS
Conc Chlorophyll a	.577	.690	NS	.635	.631	NS
Wt	1.51	2.09	NS	1.65	1.95	NS
Conc Chlorophyll b	.194	.245	.005	.257	.182	.001
Wt	.511	.693	.025	.638	.567	NS
Conc Carotenoids	.158	.179	NS	.180	.158	NS
Wt	.412	.515	NS	.447	.480	NS

analyses were significant are shown, together with results of least significant difference tests where appropriate.

Effects of pH on nutrient uptake and transport

Table 6 shows the main effects of pH of the external nutrient solution on the concentrations and total amounts of the various nutrients in shoots and roots of seedlings at the time of the second harvest of the second experiment. Significant interactions between pH levels and Zn or Cu treatments were uncommon in variance analyses; that is, there were no substantial differences in the effects of pH with different nutrient treatments. Hence, the main effects shown represent a realistic picture of pH effects.

The total amount of N in shoots and roots declined significantly with increasing pH in a manner similar to oven dry weight. There were no significant effects on concentration. This suggests that NO_3^- uptake by roots and transport to shoots kept pace with growth and was not affected by the pH of the external nutrient solution. For Cu, Zn, Fe and P there was a common tendency for significantly reduced concentrations and weights of nutrients in shoots and roots with increased pH. This suggests that both uptake by roots and transport to shoots of these nutrients declined as pH increased. For Mn, the amount retained in roots declined at pH 4; but when the total amount of Mn in shoots and roots were added to give the total amount actually taken up by the roots, this effect disappeared (results not shown).

Therefore, high pH had little effect on Mn uptake by roots, but it reduced Mn transport to shoots as with the other metals.

Metal storage in roots and effects of Zn and Cu on Zn and Cu uptake

Increasing Zn or Cu from low to high levels caused successively increased rates of uptake by roots and transfer to shoots of Zn and Cu respectively in both experiments (Tables 4,5,7). As the nutrient concentration in the external solution rose from deficient to sufficient levels, there was a marked disparity in relative nutrient concentrations in shoots and roots. Thus, in the first experiment, the concentration of Cu in roots was 1.8 times that in shoots with no Cu added to the nutrient solution, but 35 times that in shoots with 1ppm Cu (Table 4). For Zn, the effect was not as large, but the equivalent ratios increased from 1.0 to 2.8 as Zn concentration increased (Table 5). This suggests that when nutrients were present in the nutrient solution at sub-sufficient levels, roots and shoots tended to share available nutrient almost equally, but once nutrient levels rose above sufficiency, the excess available was retained in roots.

Mn and Fe, which were both supplied at supra-sufficient levels, also showed higher root concentrations than shoot concentrations (Tables 4-7). For Fe, the effect was particularly marked: shoot concentration was nearly always less than 100ppm, while root concentration was always

several thousand ppm, up to almost 8000ppm. This suggests that excesses of these metals are also stored in roots.

Interactions between nutrients for uptake and transport

There were a few isolated incidents in the first experiment where Cu and Zn interacted with other nutrients and affected their rates of uptake by roots or transport to shoots. At the lowest Cu levels in the first experiment (Table 4), there were significantly increased concentrations and total amounts of Zn and Fe retained in roots and Mn in shoots, suggesting an increased uptake rate for Zn and Fe and transport rate for Mn. The highest Cu level increased the rate of uptake of both Mn and Zn by roots. Other significant effects of Zn and Cu in the first experiment (Tables 4,5), reflected only effects on growth rate and did not give sufficient evidence to suggest there were changes in uptake or transport rates. In the second experiment (Table 7), there was little evidence that changes in Zn or Cu levels in nutrient solutions had any substantial effects on uptake or transport rates of any other nutrients.

Effects on photosynthetic pigments

Increased pH significantly reduced the fresh weight per seedling and the concentrations and total amounts of chlorophylls a and b and carotene (Table 6). This suggests that synthesis of all three pigments was reduced at higher pH

levels. Marked yellowing of seedlings occured at pH 6 and 7.

Apart from some significant changes in total amounts of photosynthetic pigments, due to differences in seedling weights with different nutrient treatments, there was little evidence that Zn or Cu levels in nutrient solutions affected pigment levels in either experiment (Tables 4,5,7). The higher Zn level increased synthesis of chlorophyll b in the second experiment (Table 7) but no similar effect was observed in the first.

DISCUSSION

Deficiency symptoms and metal requirements for growth

Hewitt (1966) suggested that 0.1ppm of both Cu and Zn in nutrient solutions is often the threshold of toxicity for plants, but up to 2ppm of either element is often not excessive. For P. radiata seedlings in this work, 0.1ppm was not excessive for either element but was just above the level required for maximum growth. Barker (1973) found that 5ppm Zn in nutrient solution was necessary for toxic symptoms to develop in P. radiata seedlings grown under similar conditions to those in these experiments. This species appears to be more tolerant of high Cu and Zn levels than some others.

In foliage, Thorne (1957) has observed that Zn deficiency symptoms occur at Zn concentrations ranging from 2-40ppm relative to dry weight, but more than 15ppm is

generally considered adequate. In these experiments, 30ppm Zn relative to dry weight in foliage was the threshold of deficiency with 70ppm in roots. For Cu, deficiency symptoms have occured in various conifers with less than 2-3ppm Cu in the foliage (Ruiter 1969, Oldenkampe and Smilde 1966, Benzian and Warner 1956). In the experiments here, growth was reduced with less than about 6ppm Cu in shoots and 20ppm in roots relative to dry weight.

Growth may decline before deficiency symptoms occur. Even with Cu in the foliage as low as 1-2ppm relative dry weight, there were few changes in appearance of seedlings, as has also been observed by Hall (1961). Certainly there were no gross distortions as observed by Ruiter (1969) in P. radiata trees several years old. The symptoms of Zn deficiency observed in these experiments were similar to those described for P. radiata seedlings by Smith and Bayliss (1942).

Growth as a function of nutrient concentration

Growth of roots and shoots of seedlings as a function of their internal Zn or Cu concentration could be explained as that part of a sigmoid curve to the right of the inflection point of the curve. When internal metal concentration was expressed relative to water content and growth logarithmically, the concentration of Zn required for adequate growth was similar in roots and shoots, but the Cu concentration required was 2-3 times higher in the root than

the shoot. Expressing metal concentrations relative to water content of shoots or roots is probably a better measure of metal availability for metabolic purposes than concentrations relative to oven dry weight. However, much of the water in the plant is metabolically inactive: the water in the vacuoles and cell walls does not take part in the metabolic processes and this water makes up the bulk of the water in the plant. The results observed here, therefore, can only be considered an initial guide to the metabolic roles of these metals.

The reasons for similar or dissimilar requirements for nutrients in root or shoot do not appear to have been elucidated for any metal. Clearly, for micronutrients, they will rest on the activities and concentrations of those metal-requiring enzymes present in roots and shoots. Availability of metals may regulate enzyme activity, or changes in concentration of metal-requiring enzymes in an organ may mean changes in requirements for the metal. Hill (1973) showed that the activity of four Cu-containing enzymes varied between roots and shoots of germinating red clover and changed over several days as germination and early growth progressed. Conversely, if there is a tendency for some metal to be retained in an inactive form in the plant, then this could lead to differences in requirement of shoot and root.

Interactions between nutrients

Much published work suggests there is usually strong competition between Cu, Zn, Mn and Fe for uptake by plants. Often these interactions have been highly complex (Smith 1962, Greenwood and Hallsworth 1960). There were only a few isolated incidents of significant interactions between these metals in these experiments. Zn, Mn and Fe uptake or transport rates increased with very low Cu levels, suggesting that these may compete more successfully for uptake or transport sites when few Cu ions are available. On the other hand, high Cu levels increased the rate of Mn and Zn uptake by roots. In general, changing Zn or Cu concentrations in the external solution did not cause the large reductions in uptake or transport of the other metals that would be expected if there were marked competition between them for uptake or transport sites.

Other work involved with metal antagonism has often used conditions very unlike those here. The uptake period was often short (Hawf and Schmid 1967, Doklya et al 1968), plants often contained excess of the elements at the beginning of the experiment (Smith and Specht 1953, Doklya et al 1968) or interactions may have occured in the soil rather than the plant (Lopez and Graham 1973, Dunne 1956). Clearly, the long term response of a plant receiving a less than adequate supply of an element could be very different to the short term response in a plant well supplied with nutrients growing in soil. These differences may have

caused the different effects observed here.

There did not appear to be any marked effect of changing Zn or Cu levels in nutrient solutions on N or P levels in roots or shoots and therefore on rates of uptake by roots or transport to shoots of NO_3^- or $HPO_4^=$. Anions and cations are generally thought to have separate uptake mechanisms (Robertson 1958, Jackson and Adams 1963), hence interactions between them would not be expected.

Nutrients in roots and shoots

When Cu, Zn, Mn and Fe were in adequate supply, concentrations of these metals in roots far exceeded those in shoots. When Cu and Zn were deficient, the root and shoot concentrations were much closer. This observation suggests that the root is the storage site for excess of these metals when they are in adequate supply. Similar effects have been observed with these metals elsewhere in a variety of species (Hawf and Schmid 1967, Lopez and Graham 1973, Smith and Specht 1953, Hill 1973), but these workers have not generally interpreted their results in this way.

Both Cu and Zn are normally bound, to a small extent, in plant cell walls (Diez-Altares and Bornemisza 1967) and this may be the cellular site at which excess metal is held. Other workers have suggested that these metals are associated with protein in the cytoplasm (Johnson and Schrenk 1963, Kositsyn and Iyoshina 1964, Kato et al 1961,

Wishnick et al 1969, Hill 1973). Further study is necessary to find how these metals are held in P. radiata.

Effects of pH

Cu, Zn and Fe uptake by roots and transport to shoots declined with increasing pH while for Mn, only the rate of transport to shoots was reduced. There were no effects of pH on N levels, but increased pH reduced P uptake and transport rates.

The effects of pH on ion uptake have been the subject of considerable study, but are still poorly understood. Robertson (1958) proposed that low pH would suppress the ionization of anions in the cell wall and the weak electrolytes of the cytoplasm. This would increase the free space available to anions and hence anion uptake. This idea has been expanded by Jackson and Adams (1963) who showed $SO_4^=$, PO_4^{3-} and Cl^- uptake by barley roots was accompanied by extrusion of OH^- ions from cells, suggesting that low pH in the external medium would promote anion uptake. Experimental evidence does not always support these suggestions. pH changes from about 5 to 9 had little effect on root uptake of NO_3^- and $H_2PO_4^-$ in tomato, lettuce and Bermuda grass (Arnon et al 1942), on NO_3^- and halide uptake by barley (Hoagland and Broyer 1937), on Cl^- uptake by beet slices (Hurd and Sutcliffe 1957) or on NO_3^- uptake by pine in this study. Other work has shown that similar pH increases have

indeed reduced NO_3^- uptake by maize roots (Van den Honert and Hooymans 1955), Cl^-, NO_3^- and PO_4^{3-} uptake by rye (Olsen 1953), Br^- uptake by barley (Jacobsen et al 1957) and $HPO_4^=$ uptake by pine in this study. Olsen (1953) suggested that reduced anion uptake at high pH was due to the higher availability of HCO_3^- ions (derived from CO_2 given off by roots), at higher pH, which competed successfully for uptake with other anions. Jackson and Adams (1963) have challenged this interpretation however. Very low pH (3-4) has reduced anion uptake by roots, but this may have been due to damage to roots at low pH (Arnon et al 1942, Jacobsen et al 1957, Hoagland and Broyer 1937).

For cations, most work has been done with K^+. A number of workers have found little effect of pH on K^+ uptake above pH 5 (Arnon et al 1942, Jacobsen et al 1957, Hoagland and Broyer 1937). All these authors found that reducing pH below this level tended to reduce K^+ uptake. Several workers have suggested that at low pH, H^+ ions compete with K^+ for uptake since H^+ ions are extruded from cells as K^+ ions are taken up (Olsen 1953, Jackson and Adams 1963) although Pitman (1970) suggested that the effect of H^+ may not be direct. Recent authors (Marre et al 1974 a,b) have proposed that changing the H^+ concentration around the cell may affect the electrochemical gradients in solution and hence cation (and anion) movement to the cell.

Very little work seems to have been done on effects of pH on uptake of heavy metals by roots and the results in

the experiment reported here do not support the suggestion that low pH reduces cation uptake by roots Results with tomato, crab grass and lettuce (Arnon and Johnson 1942) suggest very little effect of pH on growth of these species over a pH range 4-8. The results here suggested P. radiata is much more susceptible to high pH than other species. As the pH level rose in nutrient solutions, growth was much reduced, seedlings became chlorotic and synthesis of photosynthetic pigments declined. It seems likely that there was physiological damage to roots of seedlings as pH rose. This may have led to the reduced metal uptake observed which could in turn explain the decline in pigment synthesis, either directly through lack of availability of metal essential to pigment structure or through reduced activity of metal-requiring enzymes involved in pigment synthesis. The reduced pigment levels would in turn be expected to reduce growth rates.

Effects of metals on photosynthetic pigments

Neither Zn or Cu deficiencies had major effects on the photosynthetic pigments assayed. Photosynthesis requires Cu (Nason and McElroy 1963, Hill 1973) and Cu is often localized in chloroplasts (Kato et al 1961, Nelsh 1939). Results here suggested that Cu is not involved in pigment synthesis. Wacker (1962) suggested that the primary effect of Zn deficiency was reduction in protein synthesis due to derangement of RNA structure which is normally maintained by

Zn and other metals. Reduction in pigment synthesis might be a consequence of this, but is unlikely to be a primary effect of Zn deficiency.

The changes in growth rate with Zn and Cu and apparent lack of effects on N, P or photosynthetic pigments suggested that the primary effects of deficiency may be reduced activity of metal-requiring enzymes found in metabolic pathways essential to the growth process.

PAPER 2

EFFECTS OF ZN AND CU ON HEAVY METALS IN RELATION TO THE PROTEIN OF SHOOTS AND ROOTS OF *PINUS RADIATA* D.DON

ABSTRACT

P. radiata seedlings were grown for 13 weeks in solution cultures containing deficient or sufficient levels of Zn or Cu. Root and shoot material was assayed for total Cu, Zn, Mn, Fe, N, P, total water soluble amino acids and sugars. Protein was fractionated at different pH levels and metal contents determined in each fraction. Most (80 - 95%) of each metal was extracted with the protein fractions, suggesting the cell wall was only a minor repository for metals. Protein soluble in water or in tri-chloro acetic acid appeared to be the major storage site in roots for excess heavy metal. The latter fraction also appeared to contain some metal-requiring enzymes. Amino acids accumulated in shoots when Zn or Cu was deficient but protein, sugar and P metabolisms were little affected. The heavy metals did not compete for uptake by roots; Cu deficiency in fact reduced Mn uptake.

INTRODUCTION

Both Zn and Cu are co-factors to a number of plant

enzymes (Nason and McElroy 1963), many of which have been isolated and examined. Metals may be held loosely at protein surfaces or be a part of the internal protein structure (Li 1966, Gurd 1970). A number of metals are normally isolated with nucleic acids from biological material. Zn, at least, may be involved in maintaining the conformational structure of RNA and DNA (Wacker 1962).

Interactions between Zn and Cu have often been observed in plants (Smith 1962). These effects may be at the level of uptake by the root, transport to the shoot or chemically through compounds with which metals associate.

When various metals, including Zn and Cu, are present in adequate supply, their concentration in roots often vastly exceeds that in shoots. Under deficiency conditions the concentrations in both may be similar. (Hawf and Schmid 1967, Smith and Specht 1953, Lopez and Graham 1973, Hill 1973, previous paper in this work). This suggests that excess metal is stored in roots.

Few attempts have been made to bring together the physiological and biochemical aspects of Zn and Cu metabolism in plants. In the experiment described here, the effects of Zn and Cu deficiency on protein, amino acid and sugar metabolism in P. radiata seedlings were examined and related to the physiology of supply of Zn, Cu, Mn and Fe to the shoots by the root. The interactions between these elements in uptake by the root and transport to the shoot were also examined.

MATERIALS AND METHOD

Experimental

Two-week-old seedlings of P. radiata were transferred to nutrient solutions and grown in the glasshouse as described for the previous experiment. Zn- and Cu-free nutrient solutions were prepared as described previously. Pots were painted black to exclude light and prevent algal growth in the nutrient solutions.

The experiment was established as a 2^2 factorial in three randomised blocks. Cu was supplied as $CuCl_2$ at 0 or 0.1ppm Cu and Zn as $ZnSO_4$ at 0 or 0.5ppm Zn. The higher concentrations of these elements were found to be sufficient for normal growth in the previous experiment.

Harvest and chemical analysis

Seedlings were harvested 13 weeks after the treatments were applied. Three replicates were harvested for total nutrient, fresh and oven dry weight analyses and two for protein, amino acid and sugar analyses. Roots were rinsed with deionized water before separating seedlings into shoots and roots.

Material for total nutrient analysis was dried overnight in a forced draught oven at 85°C and weighed. Total N, P, Cu, Zn, Mn and Fe in dry samples were determined by the methods described in the previous experiment.

Material for protein fractionation was weighed fresh and homogenized in a 'Virtis' homogenizer in 0.05% 'Pyroneg' (Diversey, A/Asia Pty Ltd), a commercial detergent. Tests showed that neither the detergent nor the stainless steel blade of the homogenizer caused metal contamination of the homogenate. The homogenate was centrifuged at 2800g for 1hr till the supernatant, containing the water soluble protein, was clear. Successive extractions of the residue were made at 70°C for 9hr with 5% tri-chloro acetic acid (TCA) then for 17hr with 1M NaOH. Further material was precipitated from the NaOH supernatant by acidification with concentrated HCl. These fractions are referred to as the soluble fraction (i.e. soluble in 0.05% Pyroneg), TCA soluble, HCl insoluble (i.e. precipitating with HCl), alkali - acid soluble and the final residue. The HCl insoluble material was redissolved in a small amount of 1M NaOH.

Protein was estimated in each fraction, except the residue, by reduction of Folin and Ciocalteu's reagent (Lowry et al 1951) against standards of commercial bovine albumin (Calbiochem, California). Where extracted fractions had been heated, standard protein samples were heated simultaneously to standardize the assays. Total free amino acids in the soluble fraction were estimated against leucine standards by reaction with ninhydrin (Rosen 1957). Total sugars in the soluble fraction were estimated by reaction with phenol and concentrated H_2SO_4 (Bell 1955) against glucose standards. Amounts of Cu, Zn, Mn and Fe in each fraction were estimated as described in the previous experi-

ment after acid digestion of the solutions or the precipitated material.

Analysis and presentation of results

Variance of the data for fresh weight and oven dry weight per seedling, concentration and amount per seedling of total N, P, Cu, Zn, Mn and Fe (concentrations relative to oven dry weight), total protein, protein in each fraction and amino acids and sugars in the soluble fraction (concentrations relative to fresh weight) was analyzed. The concentration of each metal relative to the weight of protein (or final residue weight) in each fraction was also analyzed. Factors in the analyses were Cu level in the nutrient solution, Zn level and replicates. Results for shoots and roots were analysed separately. As in the previous experiment, all data were converted to logarithms to ensure homoscedasticity and normality of error variates: results are reported back-transformed from the logarithmic means.

Analyses of variance were also done on the proportion of the total protein found in each fraction (excluding the final residue) and the proportion of the total metal (Cu, Zn, Mn or Fe) found in each fraction (including the residue). Arcsine$\sqrt{x}$ transformations were used throughout to normalize the proportions. Generally, variances in proportions were homogeneous between shoots and roots, with or without a subsequent logarithmic transformation. Because of particular interest in the significance of differences between metal

proportions in shoots and roots, the plant part was included as a factor in these variance analyses. Where variances were not homogeneous, shoot and root data were treated separately: these instances are indicated in the results (Table 4, Fig 11).

Results are presented in a tabular form where only main effects were generally significant and as histograms where interactions between the factors of the analyses were significant. When comparisons between several means were necessary after significant differences were observed in F tests from variance analyses, the least significant difference test was used as described in the previous paper.

Table 1 shows the main effects of Zn and Cu levels in the nutrient solutions on seedling weights, concentrations and total amounts in shoots and roots of nutrients (N, P, Cu, Zn, Mn, Fe), total protein and protein in the individual fractions assayed, residue left after fractionation and sugars and amino acids extracted with the soluble fraction. In the few cases where the interaction between Zn and Cu levels were significant, the results are shown in Table 2.

Figs 1-8 show the concentrations of the heavy metals extracted in each fraction expressed relative to the weight of protein extracted in that fraction. Alongside the information for each extracted fraction are shown the factors (Zn, Cu or Zn x Cu) which were significant in the variance analyses, together with the probability levels above which they were significant and an indication of any changes in

scale used for the data in that fraction. Where the interaction was significant, results of the least significant difference test are shown with letters adjacent to the histograms. Finally, Figs 9-12 show the proportion of the total metal found in each protein fraction. As discussed above, the plant part (shoot or root) was included in these variance analyses.

RESULTS

Weight

Cu deficiency in the external solution yielded non-significant reductions in oven dry weight per seedling in shoots and roots (Table 1). Had the experiment continued longer, the deficiency would probably have significantly reduced growth. Zn deficiency significantly reduced oven dry weight per seedling of shoots, but the effect was not large enough to significantly reduce root dry weight (Table 1).

Roots of seedlings grown in solutions lacking both Zn and Cu appeared non-vigorous, lacked white tips and were quite limp. Later experiments associated these symptoms with a damping-off fungus (suspected Pythium sp), but it was not obvious if this applied here. Shoots showed some loss of vigour and colour. Table 2 shows there was a significant reduction in fresh weight per seedling of shoots and roots with the combined deficiency. This effect did not appear with oven dry weight, although there was a small, non-significant decrease (Table 2). This suggests that at the

Table 1 Main effects of Zn and Cu levels in nutrient solutions on the parameters measured in this experiment. The effects on concentrations (Conc) and total amounts (Wt) per seedling of nutrients, protein, sugars and amino acids are shown together with the level of significance (p<) of the differences between means in variance analyses. For seedling fresh weight (FW), oven dry weight (ODW) and nutrients, each value shown is the mean of six observations, and for proteins, amino acids and sugars each value is the mean of four observations. Means are back-transformed from logarithms (see text).

FW and ODW measured in g per seedling. Concentrations of N, P and protein in mg/g relative to ODW (N,P) or FW (protein) and total amounts in mg per seedling. For sugars and amino acids, concentrations in µmoles per g of FW and total amounts in µmoles per seedling. For Cu, Zn, Mn and Fe, concentrations in ppm relative to ODW and total amounts in µg per seedling. Sts = Shoots, Rts = Roots, Sol = Soluble, Alk = Alkali. * The Zn x Cu interaction was significant, see Table 2.

		Conc Zn in external solution (ppm) 0	.5	Signif icance	Conc Cu in external solution (ppm) 0	.1	Signif icance
FW	Sts	1.06	1.43	*	1.13	1.34	*
FW	Rts	.52	.67	*	.50	.70	*
ODW	Sts	.24	.29	.025	.25	.28	NS
ODW	Rts	.066	.069	NS	.064	.071	NS
N	Sts Conc	19.1	21.1	*	18.6	21.6	*
	Sts Wt	4.54	6.10	*	4.64	5.96	*
N	Rts Conc	17.9	17.3	NS	17.8	17.3	NS
	Rts Wt	1.18	1.19	NS	1.14	1.23	NS
P	Sts Conc	4.84	4.71	NS	4.48	5.09	NS
	Sts Wt	1.15	1.36	.05	1.12	1.40	.025
P	Rts Conc	6.97	8.46	.05	7.36	8.01	NS
	Rts Wt	.459	.583	.05	.470	.569	NS
Cu	Sts Conc	3.7	3.3	NS	2.0	6.0	.001
	Sts Wt	.88	.96	NS	.51	1.66	.001

Table 1 continued overleaf.....

Table 1 (continued)

		Conc Zn in external solution (ppm) 0	.5	Signif icance	Conc Cu in external solution (ppm) 0	.1	Signif icance
Cu	Conc	6.8	9.0	NS	3.3	18.6	.001
	Rts Wt	.45	.62	NS	.21	1.32	.001
Zn	Conc	15.5	67.4	.001	33.7	31.1	NS
	Sts Wt	3.7	19.5	.001	8.4	8.6	NS
Zn	Conc	23.	321.	.001	95.	77.	NS
	Rts Wt	1.5	22.1	.001	6.1	5.5	NS
Mn	Conc	203.	216.	NS	172.	256.	.005
	Sts Wt	48.4	62.6	NS	43.0	70.5	.01
Mn	Conc	175.	314.	NS	96.	572.	.001
	Rts Wt	11.5	21.6	NS	6.1	40.7	.001
Fe	Conc	134.	112.	NS	124.	121.	NS
	Sts Wt	32.0	32.4	NS	31.0	33.4	NS
Fe	Conc x.1	775.	795.	NS	865.	711.	NS
	Rts Wt	511.	548.	NS	553.	506.	NS
Sol amino acids	Conc	28.2	17.2	*	24.5	19.9	*
	Sts Wt	28.8	25.2	*	28.0	25.9	*
Sol amino acids	Conc	6.78	6.63	NS	4.96	9.07	NS
	Rts Wt	3.37	4.40	NS	2.27	6.53	NS
Sol sugars	Conc	9.12	8.01	.05	9.50	7.70	.01
	Sts Wt	9.3	11.7	NS	10.9	10.0	NS
Sol sugars	Conc	4.45	3.78	NS	4.57	3.68	NS
	Rts Wt	2.21	2.51	NS	2.09	2.65	NS

Table 1 continued overleaf.....

Table 1 (continued)

		Conc Zn In external solution (ppm) 0	.5	Signif icance	Conc Cu In external solution (ppm) 0	.1	Signif icance
Total protein	Conc Sts	46.1	30.0	.001	39.7	34.6	NS
	Wt	47.1	43.5	NS	45.4	45.1	NS
Total protein	Conc Rts	20.4	12.6	.001	17.8	14.4	NS
	Wt	10.1	8.4	NS	8.2	10.4	NS
Sol protein	Conc Sts	13.3	9.4	NS	14.2	8.8	NS
	Wt	13.7	13.7	NS	16.2	11.5	NS
Sol protein	Conc Rts	8.84	6.25	.05	8.65	6.39	.05
	Wt	4.39	4.15	NS	3.96	4.60	NS
TCA sol protein	Conc Sts	4.09	2.00	.025	3.52	2.33	.05
	Wt	4.18	2.92	NS	4.02	3.03	NS
TCA sol protein	Conc Rts	1.56	.99	.025	1.52	1.02	.025
	Wt	.775	.660	NS	.696	.736	NS
HCl insol protein	Conc Sts	23.8	15.3	.001	18.1	20.1	NS
	Wt	24.4	22.4	NS	20.7	26.3	NS
HCl insol protein	Conc Rts	4.00	2.87	.01	3.80	3.02	.025
	Wt	1.99	1.91	NS	1.74	2.18	NS
Alk-acid protein	Conc Sts	4.10	2.47	.05	3.31	3.06	NS
	Wt	4.19	3.61	NS	3.79	3.99	NS
Alk-acid protein	Conc Rts	5.81	2.41	.01	3.66	3.82	NS
	Wt	2.88	1.60	NS	1.68	2.75	NS
Residue	Conc Sts	85.0	87.3	NS	88.8	83.5	NS
	Wt	83.	121.	NS	107.	119.	NS
Residue	Conc Rts	40.0	39.1	NS	39.1	40.0	NS
	Wt	41.2	56.2	NS	44.3	53.2	NS

Table 2 Data similar to those of Table 1, but showing the effects of the interaction between Zn and Cu levels in the nutrient solution on particular parameters. Means which did not differ significantly in the least significant difference test, have similar letters beside them.

		Zn and Cu levels in solution (ppm)				Significance
		Zn 0 Cu 0	Zn 0.5 Cu 0	Zn 0 Cu 0.1	Zn 0.5 Cu 0.1	(p<)
FW	Sts	.92	1.39a	1.22a	1.47a	.05
FW	Rts	.34	.72a	.79a	.62a	.01
ODW	Sts	.22	.28	.26	.29	NS
ODW	Rts	.058	.071	.075	.067	NS
N	Conc	16.6	20.7a	21.8a	21.4a	.05
	Sts Wt	3.66	5.89a	5.64a	6.31a	.05
Sol amino acids	Conc	24.1a	24.9a	33.0a	12.0	.05
	Sts Wt	21.8bc	36.0ab	38.1a	17.6c	.025

time of harvest, the main loss due to the combined Zn and Cu deficiency had been in turgor not dry weight, which implies growth was not severely impaired until shortly before the harvest. Further results were considered with these effects in mind.

Total phosphorus and soluble sugars

There were no significant effects of Zn or Cu deficiency on transport of P to shoots or rate of synthesis of soluble sugars in shoots and transfer to roots (Table 1). Zn deficiency significantly reduced P uptake by roots, but Cu deficiency had no significant effects (Table 1)

Total N, amino acids and protein

Total N in the seedlings was little affected by the individual Zn or Cu deficiencies in shoots or roots (Table 1). The combined Zn and Cu deficiency reduced total N in shoots (Table 2), but had no similar effect in roots.

There were no significant effects of either metal deficiency or the combined deficiency on the amount per seedling of total extracted protein (the sum of the protein in each fraction) (Table 1). There were some significant effects on total protein concentration, but these reflected only changes in seedling weight with metal deficiencies. That is, there were no significant effects of metal deficiency on total protein synthesis. Similarly, there were no

substantial effects of solution Zn or Cu levels on levels of protein found in the individual fractions (Table 1).

Both Zn and Cu deficiencies significantly increased the concentration and total amount per seedling of soluble amino acids in shoots (Table 2) but had no significant effects in roots (Table 1).

Table 3 The proportions of the total protein of shoots or roots found in different extracted fractions (%). The results show the main effects of plant part (shoot or root) in variance analysis. Each result is meaned over eight observations and is back-transformed from a log (arcsin($\sqrt{x}$)) transformation (see text). Separate analyses were done for each fraction. The significance (p<) of the differences between proportions found in shoots and roots is also shown.

	Protein fraction			
	Soluble	TCA soluble	HCl insoluble	Alkali-acid soluble
Shoots	30.4	7.7	51.8	8.6
Roots	46.8	7.6	20.8	23.1
Significance (p<)	.005	NS	.05	.001

This suggests both deficiencies led to a build up of soluble amino acids in shoots but had little effect in roots. The combined Zn and Cu deficiency did not show as marked an increase in shoots as the individual deficiencies (Table 2) but this may be an effect of disturbed metabolism due to fungal effects on these seedlings (see above).

Most (70-80%) of the protein was found in the soluble and HCl insoluble fractions (Table 3). The HCl insol-

uble protein was present in higher proportion in shoots than roots, while the soluble and alkali-acid soluble protein were significantly more prominent in roots than shoots. TCA soluble protein was present in similar proportions in both roots and shoots.

Copper

Cu deficiency significantly reduced the concentration and total amount of Cu in shoots and roots (Table 1), showing reduced Cu uptake by roots and transport to shoots. The seedlings in Cu deficient solutions had Cu concentrations below those required for unimpaired growth (6ppm in shoots and 20ppm in roots as found in the previous experiment). At the higher Cu level, Cu concentration in roots far exceeded that in shoots (Table 1) while at the Cu deficient level, the concentrations in shoots and roots were similar, although slightly higher in roots.

The proportion of the total Cu found in the TCA soluble fraction increased significantly in roots and non-significantly in shoots with Cu deficiency (Fig 9). There were no significant effects in other fractions. About 90% of the total Cu in roots and shoots was found in the extracted fractions and relatively little in the residue (Table 4). In shoots, a significantly higher proportion of the total Cu was found in the HCl insoluble fraction and residue than in the roots, while the reverse was true in other fractions (Table 4).

Table 4 The main effect of plant part (shoot or root) on the proportion (%) of the total metal found in the extracted fractions (see discussion in the text of the validity of the analyses). Each value is the mean of eight observations. The significance of the differences between means in variance analysis is also shown. SOL = Soluble fraction, TCA = TCA soluble fraction, HCL = HCl insoluble fraction, A-A = Alkali-acid soluble fraction, RES = Residue, Sts = Shoots, Rts = Roots.

		Protein fraction				
		SOL	TCA	HCL	A-A	RES
Cu	Sts	20.0	11.5	41.4	12.6	14.7
	Rts	29.8	31.6	15.4	20.1	4.1
Significance (p<)		.05*	.01*	.001	.005	.001
Zn	Sts	67.0	20.2	4.4	5.8	2.9
	Rts	54.0	31.9	2.1	7.0	3.2
Significance (p<)		.025	.025	.025	NS	NS
Mn	Sts	86.3	10.7	1.0	0.7	1.1
	Rts	73.9	20.0	0.1	2.0	2.9
Significance (p<)		.01*	.001	.01*	.01*	.005
Fe	Sts	15.9	54.9	0.1	0.1	29.9
	Rts	14.8	67.6	0.2	3.2	14.2
Significance (p<)		NS	.05	NS	.01	.025

* Variances between data for roots and shoots were not homogeneous. The technique for analysis of heteroscedastic data given by Sokal and Rohlf (1969) was used.

Figs 1-12 Concentrations of metals relative to protein in extracted fractions and proportions of metals in extracted fractions (see discussion in text). SOL = Soluble fraction, TCA = TCA soluble fraction, HCL = HCl insoluble fraction, A-A = Alkali-acid soluble fraction, RES = Residue. Concentrations of Zn in nutrient solutions shown as Zn 0 (i.e. 0ppm) or Zn .5. Significance of effects shown as, e.g. Cu <.05, i.e. the main effect of Cu was significant with $p<.05$.

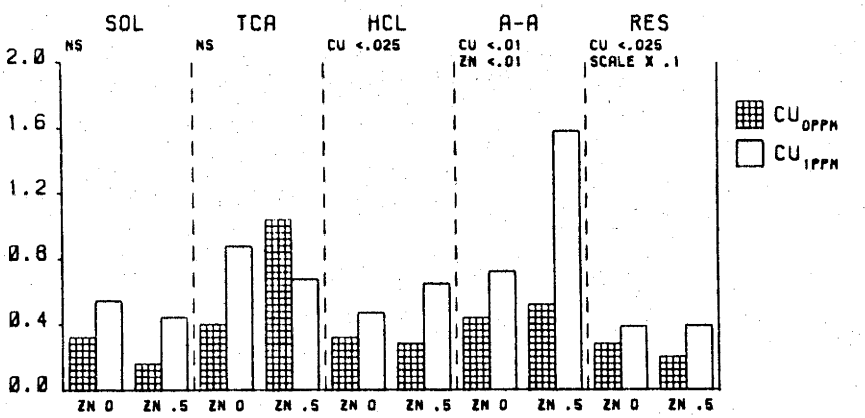


FIG 1 CONC. OF CU IN SHOOTS RELATIVE TO PROTEIN OR RESIDUE WEIGHT (PPM $X10^{-2}$)

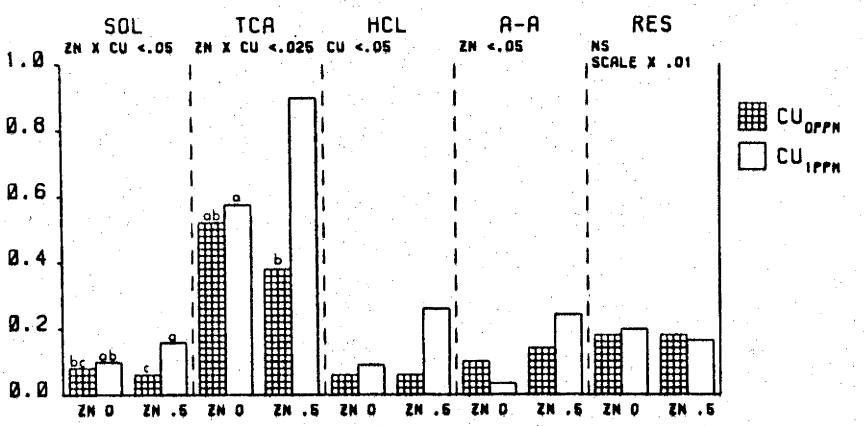


FIG 2 CONC. OF CU IN ROOTS RELATIVE TO PROTEIN OR RESIDUE WEIGHT (PPM $X10^{-3}$)

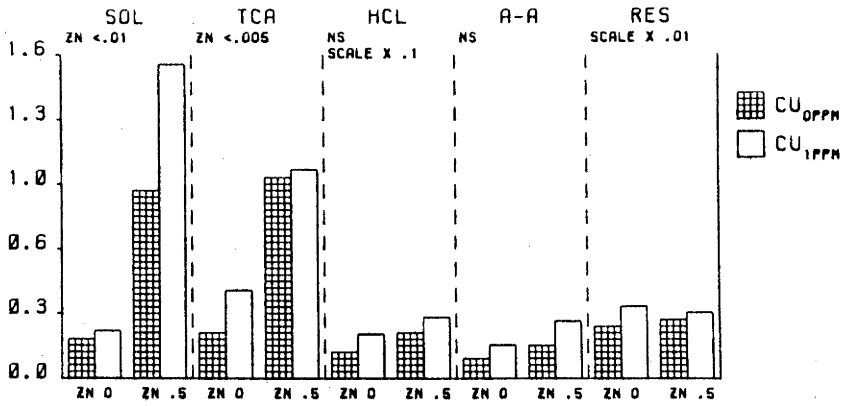


FIG 3 CONC. OF ZN IN SHOOTS RELATIVE TO PROTEIN OR RESIDUE WEIGHT (PPM X10^{-3})

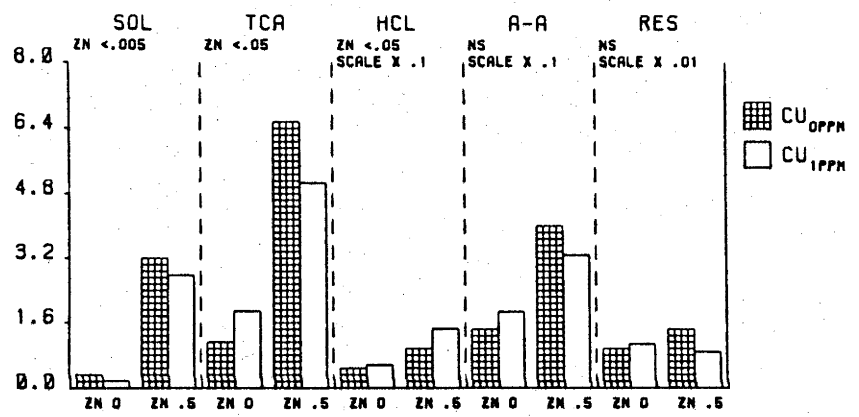


FIG 4 CONC. OF ZN IN ROOTS RELATIVE TO PROTEIN OR RESIDUE WEIGHT (PPM X10^{-3})

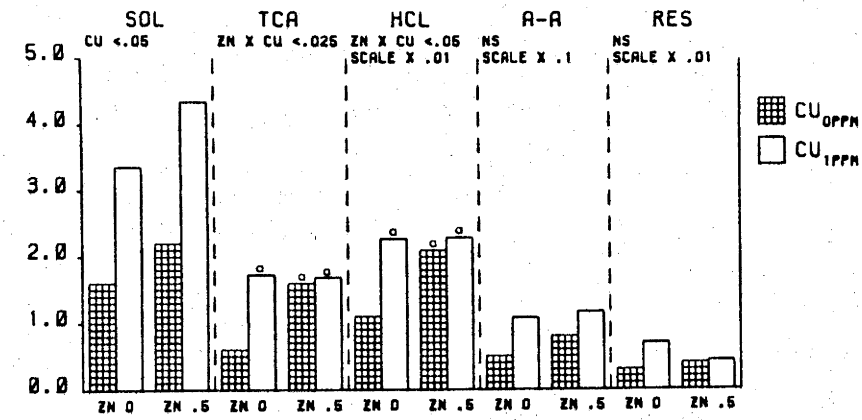


FIG 5 CONC. OF MN IN SHOOTS RELATIVE TO PROTEIN OR RESIDUE WEIGHT (PPM X10^{-3})

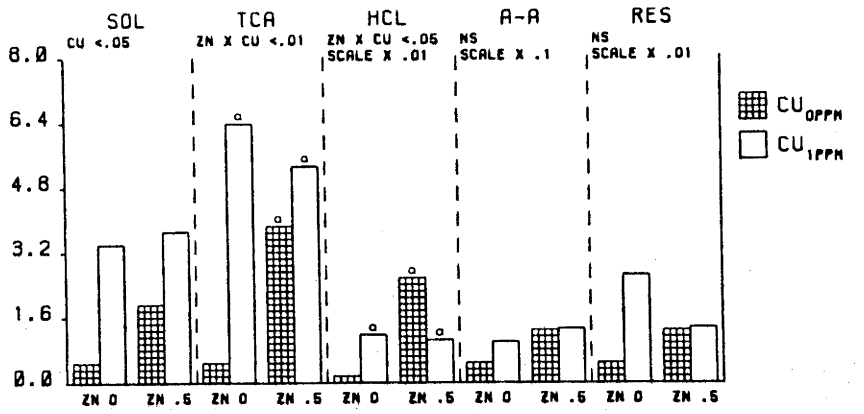


FIG 6 CONC. OF MN IN ROOTS RELATIVE TO PROTEIN OR RESIDUE WEIGHT (PPM X10^{-3})

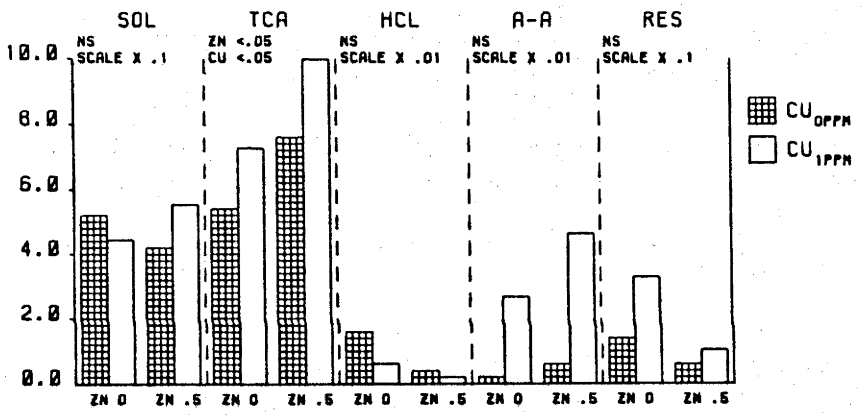


FIG 7 CONC OF FE IN SHOOTS RELATIVE TO PROTEIN OR RESIDUE WEIGHT (MG/G)

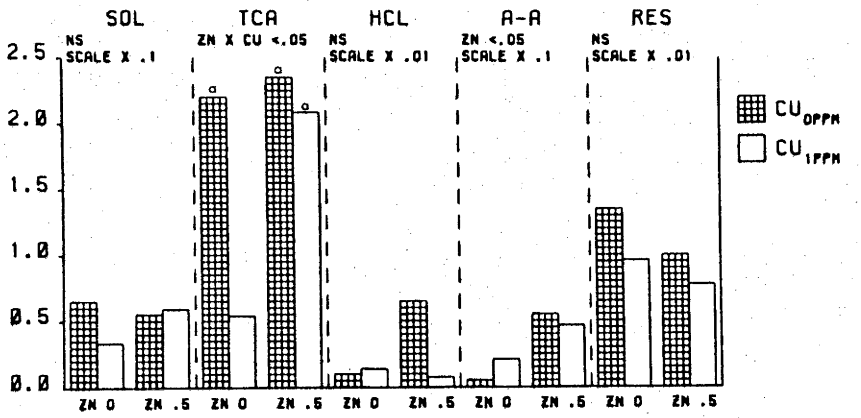


FIG 8 CONC. OF FE IN ROOTS RELATIVE TO PROTEIN OR RESIDUE WEIGHT (MG/G X10^{-2})

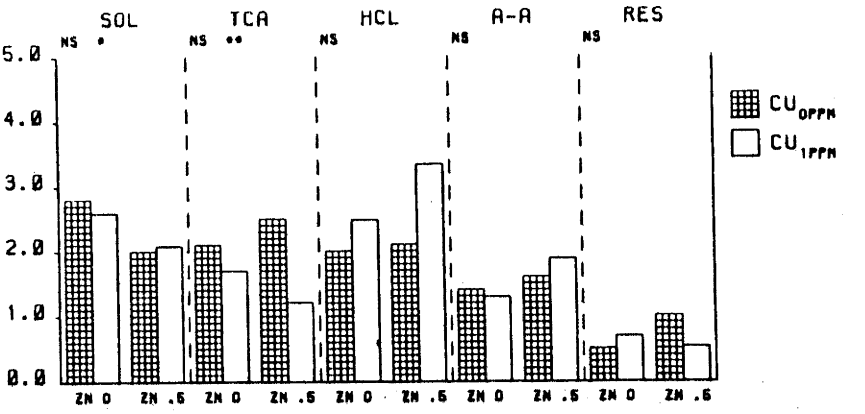


FIG 9 PROPN. OF TOTAL CU OF SHOOTS AND ROOTS IN VARIOUS EXTRACTED FRACTIONS (% X10)

* Variance of data between roots and shoots was not homogeneous. Separate analyses were done for roots and shoots. Data shown, however, were meaned over shoots and roots.

** There was a significant effect of Cu in shoots, where Cu deficiency significantly increased the proportion of Cu.

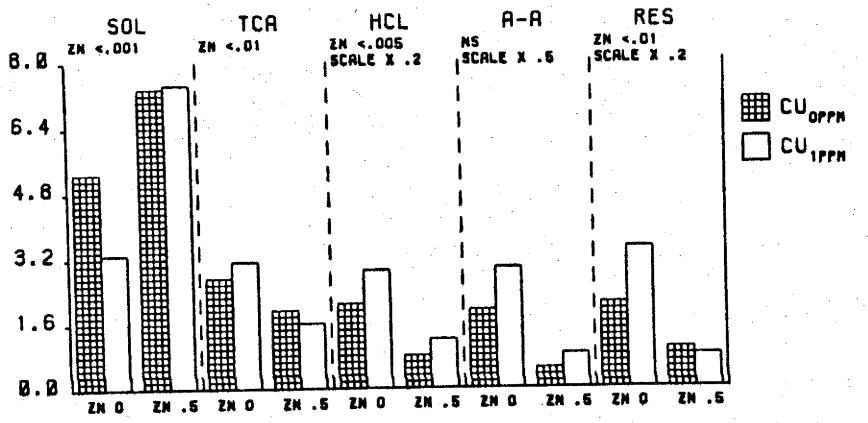


FIG 10 PROPN. OF TOTAL ZN OF SHOOTS AND ROOTS IN VARIOUS EXTRACTED FRACTIONS (% X10)

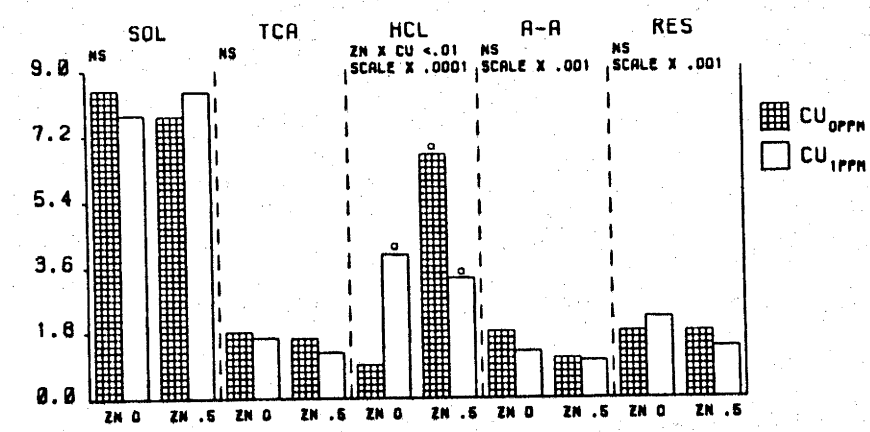


FIG 11 PROPN. OF TOTAL MN OF SHOOTS AND ROOTS IN VARIOUS EXTRACTED FRACTIONS (% X10)

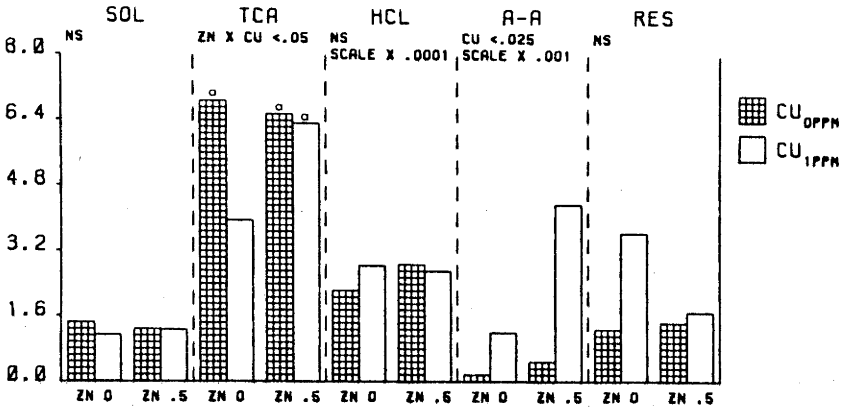


FIG 12 PROPN. OF TOTAL FE OF SHOOTS AND ROOTS IN VARIOUS EXTRACTED FRACTIONS (% X10^3)

Cu deficiency reduced the concentration of Cu relative to the protein in the HCl insoluble fraction in shoots and roots and relative to the residue in shoots (Figs 1,2). Zn deficiency reduced the concentration of Cu relative to the alkali-acid soluble protein in roots (Fig 2). Both Zn and Cu deficiencies reduced the concentration of Cu relative to the protein in the alkali-acid soluble fraction in shoots and the soluble and TCA soluble fractions in roots. The effects in the latter fractions applied only if the other metal were in adequate supply (Figs 1,2).

Zinc

Zn deficiency significantly reduced the total amount and concentration of Zn in shoots and roots (Table 1), showing reduced Zn uptake by roots and transport to shoots. Seedlings in Zn deficient solutions had Zn concentrations in

shoots and roots below the levels required for unimpaired growth (30ppm in shoots and 70ppm in roots as found in the previous experiment). Concentration of Zn in roots was slightly higher than in shoots when Zn was deficient, but was very much greater in roots than shoots when Zn was sufficient (Table 1).

When Zn was deficient, the proportion of Zn in the soluble fraction decreased significantly and increased in all other fractions in both shoots and roots (Fig 10). In shoots, a significantly higher proportion of the total Zn was found in the soluble and HCl insoluble fractions than in roots, while in the TCA soluble fraction more Zn was present in the root fraction than the shoot (Table 4). The bulk (about 85%) of the total Zn of shoots and roots was extracted with the soluble and TCA soluble fractions.

Concentration of Zn relative to the soluble and TCA soluble protein was significantly reduced with Zn deficiency in shoots and roots (Figs 3,4), even though the proportion of Zn in the TCA soluble fraction had increased with Zn deficiency (Fig 10). In the HCl insoluble fraction, the reduction occurred only in roots (Fig 4).

Cu deficiency had no significant effects on total Zn (Table 1), or Zn held in various fractions (Figs 3,4,10).

Manganese

Cu deficiency significantly reduced the total

amount and concentration of Mn in shoots and roots (Table 1), suggesting reduced Mn uptake by roots and transport to shoots. Root Mn concentration substantially exceeded shoot concentration when Cu was in adequate supply, while shoot concentration exceeded root concentration when Mn availability was reduced by Cu deficiency (Table 1).

About 95% of the Mn was extracted with the soluble and TCA soluble fractions (Table 4). In the TCA and alkali-acid soluble fractions, and in the residue, a significantly higher proportion of the total Mn was found in roots than shoots, while more was in shoots than roots in the soluble fraction (Table 4). The only significant effect of Zn or Cu deficiencies on the proportion of Mn found in the various fractions, was a reduction in the HCl insoluble fraction with the combined deficiency (Fig 11).

Cu deficiency significantly reduced the concentration of Mn relative to the protein in the soluble fraction in shoots and roots (Figs 5,6). The combined Zn and Cu deficiency significantly reduced the concentration of Mn relative to the protein in the TCA soluble and HCl insoluble fractions, while the individual deficiencies had no effects (Figs 5,6).

Iron

At the level of Fe supplied in the nutrient solutions, concentration of Fe in roots far exceeded that in

shoots - about 8000ppm compared with about 120ppm relative to oven dry weight (Table 1).

About 60% of the total Fe was extracted with the TCA soluble fraction, about 15% with the soluble fraction and 20% with the residue in both shoots and roots (Table 4). In the TCA soluble fraction, the proportion of the total Fe found in roots was significantly higher than in shoots, while in the residue the proportion in shoots was higher (Table 4).

Neither Zn nor Cu deficiencies had significant effects on the rate of Fe uptake by roots or transport to shoots (Table 1). However, Zn deficiency significantly reduced the proportion of Fe in the TCA soluble fraction in shoots and roots, but only when Cu was in adequate supply (Fig 12): this caused a significant decrease in Fe concentration relative to TCA soluble protein in roots (Fig 8). In shoots, both Zn and Cu deficiencies significantly reduced the Fe concentration relative to the protein in the TCA soluble fraction (Fig 7). Cu deficiency significantly reduced the proportion of Fe in the alkali-acid soluble fraction (Fig 12), but this had no significant effects on Fe concentration relative to the protein in that fraction (Figs 7,8), although there was a non-significant decrease in shoots. Zn deficiency did not affect the proportion of Fe in the alkali-acid soluble fraction (Fig 12), but did significantly reduce the concentration of Fe relative to the alkali-acid soluble protein in roots (Fig 8).

DISCUSSION

Metals in relation to cell protein

The bulk of all four metals assayed was found in the extracted fractions rather than in the residue which usually contained much less than 15% of the Cu, Zn and Mn of shoots and roots and less than 30% of the Fe (Table 4). Other work with a number of plants has shown that Cu and Zn are normally associated with protein in the cell. From 80-100% of the Zn in plants has often been found in soluble cell fractions or associated with soluble proteins (Kositsyn and Iyoshina 1964, Johnson and Schrenk 1963, Diez-Altares and Bornemisza 1967, Ozanne 1955). Much leaf Cu is associated with certain proteins (Kato et al 1961, Wishnick et al 1969, Neish 1939) while Hill (1973) found this was also true of roots. On the other hand, both Cu and Zn are normally found to a small extent in plant cell walls (Diez-Altares and Bornemisza 1967). Kositsyn and Iyoshina (1964) found that when fractionating Zn from tomato leaf, the supernatant had very strong absorption properties for Zn, suggesting that some Zn normally resident in cell walls might have been extracted from the walls with the supernatant. Loosely bound metals may also be very easily removed from protein by treatment with mineral acids (Haurowitz 1963). It is, therefore, only with reservation that one can suggest that the protein fractions extracted here originally contained the metals assayed in them. The results support the general view that most plant metal is associated with protein or other cell constituents and very little is retained in the wall.

When the various metals were in above sufficient supply, there was a very marked increase in metal concentration in roots. When Cu, Zn or Mn availability was low, root and shoot metal concentrations were much closer (Table 1). Increased metal concentration in roots with excess metal has been observed elsewhere (Hawf and Schmid 1967, Smith and Specht 1953, Lopez and Graham 1973, Hill 1973) and when availability has been reduced, root concentration has declined much more than shoot concentration (Polson and Adams 1970, Wood and Sibley 1950). These results suggest that the roots act as the storage location for excess metals when they are in supra-sufficient supply.

If roots tend to retain excess nutrient, it is likely that some part of the cell system would act as a store for the excess. In Zn and Cu resistant clones of the grass Agrostis tenua, the cell wall retained excess metal (Turner 1970). In the experiment reported here, none of the protein fractions extracted showed all the properties that might be expected of a site that stored excess metal. However, protein in the soluble and TCA soluble fractions showed several of these properties which are discussed below.

Firstly, with metal deficiency, the concentration of a metal expressed relative to the weight of the storage fraction would be expected to decline more sharply than in other fractions. Zn deficiency caused a reduction in Zn concentration relative to the protein in both the soluble and TCA soluble fractions; the effect applied in both shoots and

roots (Figs 3,4). Cu deficiency reduced both Cu and Mn availability to the seedlings, but it was only in the soluble fraction that the concentration of both these metals relative to the protein in that fraction declined significantly (Figs 1,2,5,6).

Secondly, as the availability of a metal increases above deficiency levels, the proportion of the metal found in a metal storage fraction would be expected to increase, relative to other fractions. With Cu or Zn sufficiency, the proportion of Cu or Zn, respectively, held in the TCA soluble fraction decreased, rather than increased; in the case of Cu, the decrease occurred only in roots (Figs 9,10). This suggests that this fraction may contain the Zn and Cu requiring enzymes, since retention of essential metal in that fraction under deficiency conditions would clearly be advantageous to the plant. On the other hand, in the soluble fraction, the proportion of Zn extracted with that fraction decreased with Zn deficiency as might be expected of a metal storage fraction. But there were no similar effects in this fraction with Cu or Mn under conditions of Cu deficiency (Figs 9,11).

Thirdly, a storage fraction might be expected to hold a substantial proportion of the total metal of the root, particularly when the metal was in above adequate supply. As well, it might be expected to hold a greater proportion of the total metal of the root than that of the shoot. Both the soluble and the TCA soluble fractions consistently contained

substantial proportions of all four metals (Table 4). However, only the TCA soluble fraction consistently held a higher proportion of metal in roots than shoots; this did not differ under different conditions of metal availability.

All these properties assume that the availability of the storage fraction is not altered by availability of metal: in this experiment, changes in metal availability did not substantially alter the levels of proteins in any of the extracted fractions.

Whilst these results are not conclusive, they do suggest that the protein in both the soluble and TCA soluble fractions behaves in many ways as might be expected of a metal storage fraction. There is, however, one further difficulty with the interpretation of these results. The extraction procedure used in this experiment was similar to that of Schneider (1945): it is likely that the TCA soluble fraction would have contained the nucleic acids as well as protein. Martin (1966) found 10-11mg/g of fresh weight of nucleic acid (RNA + DNA) in root tips of a wide range of plants. Roots in this experiment had a total of about 17mg/g fresh weight of protein of which only about 10% was in the TCA soluble fraction (Table 3). Nucleic acids normally contain metals essential to their structure (Wacker and Vallee 1959, Wacker 1962). Thus, because amounts of nucleic acids and protein in the plant may be comparable, it is feasible that at least some, and possibly all, of the metal found in the TCA soluble fraction could have been associated

with nucleic acids. The discussion above suggests that protein is more likely as the metal store, but further work is necessary to satisfy this point.

There were no effects of Zn or Cu deficiency on levels of total protein, or the protein extracted in the various fractions. This is in general agreement with other work. Wood and Womersley (1946) showed little effect of Cu deficiency on oat protein, while Nason (1952) showed a change in protein quality but not quantity in Cu deficient tomato leaves. Zn deficiency has caused changes in protein quality and quantity (Nason and McElroy 1963, Nason et al 1965), but the total protein balance seems to have been little studied. The lack of major effects of the deficiencies on protein does not support the suggestion that Zn or Cu deficiency symptoms in leaves may be caused by binding of metals to excess protein in roots (Ozanne 1955, Gilbert 1951).

Both Zn and Cu deficiencies increased the level of soluble amino acids in shoots but not roots, but the combined deficiciency had no significant effects. Increase in amino acid synthesis has often been observed with Zn deficiency (Possingham 1956, Steinberg 1956, Wacker 1962) and Cu deficiency (Possingham 1956), although in Cu deficient tobacco leaves some amino acids increased, while others decreased (Steinberg 1956). Changes in rate of protein synthesis have been ascribed to these changes (Wacker 1962): this seems unlikely in this experiment where no effects on total protein were observed. Hill (1973) suggested that metals may complex

with amino acids during transport to shoots. The highly polar nature of amino acids makes this chemically feasible (Brown 1963): amino acids have been found in the xylem stream of northern hardwoods (Bollard 1958) and in root saps in eucalypts (Wilson and Bachelard 1975). If amino acids transport metals, a build up of amino acids in shoots would seem possible as a plant response to attempt to transport more metal to shoots. However, there seems to be general agreement that these metals (except Fe) exist as free cations in the xylem stream (Tiffin 1967, Biddulph 1959, Sutcliffe 1962).

The overall total N balance in shoots was not affected in a similar way to amino-N by Zn or Cu deficiency (Table 2). This suggests other N compounds (e.g. nucleic acids), not measured in this experiment, may have been affected, to balance the rise in amino-N in shoots. N balance of roots was little affected by the metal deficiencies.

Interactions between metals

Cu deficiency reduced uptake of Cu and Mn by roots and transport to shoots. Zn deficiency reduced uptake and transport of Zn. There were no effects of Zn or Cu on rates of uptake or transport of Fe, no effects of Zn on Cu or Mn and no effects of Cu on Zn. This is in direct contrast to much published work, where antagonisms to uptake by roots or transport to shoots between these four metals have commonly

been observed, often of a highly complex nature (Smith 1962, Greenwood and Hallsworth 1960). However, the uptake period examined has often been short (hours to a few days) (Hawf and Schmid 1967, Schmid et al 1965, Lingle et al 1963, Bowen 1969, Polson and Adams 1970, Ishizuka and Ando 1968). Other workers have observed interactions in plants after soil applications of nutrients (Dunne 1956, Fuehring and Soofi 1964). However, nutrient interactions may occur in the soil, not the plant (Lopez and Graham 1973). Under these conditions, a plant response may reflect only a soil response Clearly, the physiological response of a plant may be very different when a metal is in short supply than when it is adequate. Preferential uptake of an element might occur despite the presence of other ions in solution which would otherwise compete with it successfully. The results in the experiment reported here indicated that, in the long term, uptake of Cu and Mn may be synergistic in that Cu deficiency may interfere with the normal uptake mechanism of Mn. This could be caused by a requirement in the Mn uptake mechanism for Cu.

Both Zn and Cu deficiencies caused reductions in concentrations of the other metals relative to the protein in several extracted fractions in both shoots and roots (Figs 1-8). These effects are in contrast to the effects on total nutrient levels where inter-metal competition was not extensive (Table 1). Enzymes are not always highly specific for the metals essential for their activity that accumulate on their surfaces at active sites (Vallee 1962). Thus,

changes in availability of one metal might cause changes in the concentration of another relative to a protein fraction: but under these circumstances, increases in the concentration of the second metal might be expected, not decreases as observed here. Further work is necessary to explain the effects observed.

Effects on other metabolites and growth

Rate of synthesis and transport to roots of soluble sugars was little affected in this experiment by Zn or Cu deficiency. Nason and McElroy (1963) suggested Cu is largely involved in photosynthesis as an enzyme co-factor. In this experiment, Cu deficiency was not sufficiently severe or did not persist long enough to prevent sugar production. Nevertheless, growth was reduced with metal deficiencies, suggesting that metabolic effects, other than effects on photosynthesis, were the primary effects of the deficiencies.

There was no build up of total P in this experiment with Zn deficiency. Increases in organic phosphate (Hewitt 1963) or organic polyphosphate (Wacker 1962) have been observed elsewhere with Zn deficiency. Cu deficiency had no effects on total P.

Under the conditions of this experiment, it appears that reduced growth was the primary metabolic effect of Zn or Cu deficiency, probably due to reduction in enzyme activity with reduced metal availability. More severe deficiencies,

or a longer growth period, might have induced the other symptoms which have been observed elsewhere.

PAPER 3

EXTRACTION AND ASSAY OF PROTEIN, NUCLEIC ACIDS AND HEAVY METALS FROM TISSUE OF *PINUS RADIATA* D.DON

ABSTRACT

The Schmidt-Thannhauser and Schneider techniques for nucleic acid extraction were tested with homogenates of *P. radiata* tissue. Both techniques were unsuccessful. It was impossible to assay RNA or DNA either colorimetrically or with UV spectrophotometry because of interference from other substances extracted with the nucleic acids. These techniques formed the basis for a protein fractionation procedure based on solubility of protein at different pH levels. Extracted protein could be precipitated from most fractions. The heavy metals Cu, Zn, Mn and Fe seemed to be closely associated with the protein in these fractions.

INTRODUCTION

The biochemistry of heavy metals in plants is closely related to protein and nucleic acid metabolism. Many proteins require metals as cofactors and nucleic acids contain metals in their physical structure. Metals may be held in plants in association with protein (Johnson and Schrenk 1963, Kositsyn and Iyoshina 1964, Kato et al 1961,

Wishnick et al 1969, Hill 1973). In the previous paper, attempts were made to find the relation between heavy metals and protein in P. radiata tissue. Problems of distinguishing between protein and nucleic acids in extract fractions were discussed. This paper describes attempts to extract and assay protein and nucleic acids from P. radiata tissue and find the metal contents of the extracts.

Nucleic acids have generally been extracted from biological material using either the Schmidt-Thannhauser or Schneider techniques (Hutchinson and Munro 1961). Both techniques first involve removal of interfering substances during homogenization of tissue in cold acid. Lipids are then removed with organic solvents. In the Schmidt-Thannhauser procedure, the residue is then incubated with alkali. This renders the DNA acid-insoluble so on acidification of the digest DNA is precipitated and RNA remains in the supernatant. In the Schneider procedure, both DNA and RNA are extracted simultaneously with hot acid. The two nucleic acids are then assayed with different colorimetric techniques. Another variation is the method of Ogur and Rosen (1950) who extracted RNA first with cold acid then DNA by further incubation with hot acid. Hutchinson and Munro (1961) have extensively reviewed these procedures. In developing techniques here, their recommendations have generally been adopted.

Extraction of different protein fractions based on their solubility at different pH levels is common (Haurowitz

1963). Since the techniques of nucleic acid extraction require acid or alkali solutions, they may yield protein fractions at the same time. These techniques formed the basis of the protein fractionation technique developed in this work.

ASSAY TECHNIQUES

Protein

The reduction of Folin and Ciocalteu's phosphomolybdic-phosphotungstic reagent by copper treated protein is a sensitive and convenient method for protein assay (Lowry et al 1951). This method was used throughout this work. Protein content of samples was estimated against standards of bovine albumin (Calbiochem Calif.).

Since protein extraction procedures sometimes involved hot extracts, the effects of heating protein on the subsequent reaction with Folin and Ciocalteu's reagent was examined. Standard solutions of bovine albumin were heated at different temperatures and for different time periods. The solutions were then serially diluted, the Lowry procedure followed and the absorbance of the solutions measured at 750mμ. The results for several of the tests are shown in Fig 1. Both increased temperature and increased incubation period reduced absorbance, the effect increasing as protein concentration increased. With the most severe treatment (80°C for 20.5 hr), the curve flattened off at the highest protein concentrations. For routine analyses, protein

standard samples were heated at the same time as test solutions.

Nesslerization has often been used for protein assay. This involves the treatment of protein with strong acid and the measurement of total N in the resultant solution. N content is then converted to protein content by an appropriate conversion factor. This method was tested, as described later, for protein assay. Concentrated H_2SO_4 containing 200g/l K_2SO_4 and 1g/l Se (Jackson 1958) was used for digestion. Colorimetric estimation of N used the reaction of NH_4^+ with sodium phenoxide in the presence of sodium hypochlorite to yield an unknown blue product (Anon 1971).

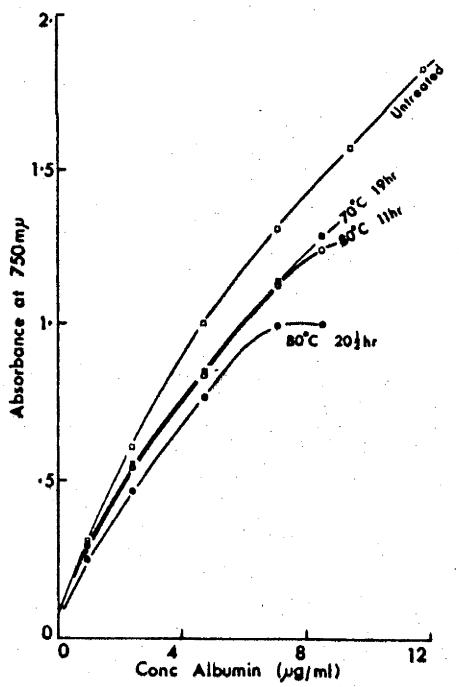


Fig 1 Effect of time and temperature of heating standard samples of bovine serum albumin on response in the Lowry protein assay. Absorbance measured at 750mμ.

Nucleic acids

Colorimetric method for DNA

The diphenylamine procedure for DNA estimation involves the reaction of diphenylamine with deoxyribose residues of DNA released by heating with glacial acetic acid and concentrated H_2SO_4 to yield a coloured product. The technique used was that of Burton (1956). Standards were of DNA calf thymus (Calbiochem Calif.).

Colorimetric method for RNA

Hydrolysis of RNA at 100°C yields furfural which gives a green colour with orcinol (Hutchinson and Munro 1961). The orcinol method of Schneider (1957) for RNA assay was used here, but orcinol was not recrystallized before use. Standards were of RNA purified from Torula (Calbiochem Calif.).

Ultra-violet light determination of nucleic acids

Both DNA and RNA may be assayed by the absorbance of their purine and pyrimidine bases at 260mμ (Hutchinson and Munro 1961). Interference from protein degradation products extracted with nucleic acids has often made this impossible. Techniques to minimise the amount of protein extracted with the nucleic acid fractions have been tested and methods to eliminate the interference by measuring at two wavelengths developed. The effectiveness of these techniques has varied

with different extracts. Attempts were made here to assay extracted nucleic acid by UV light absorption.

EXTRACTION AND ASSAY OF PROTEIN

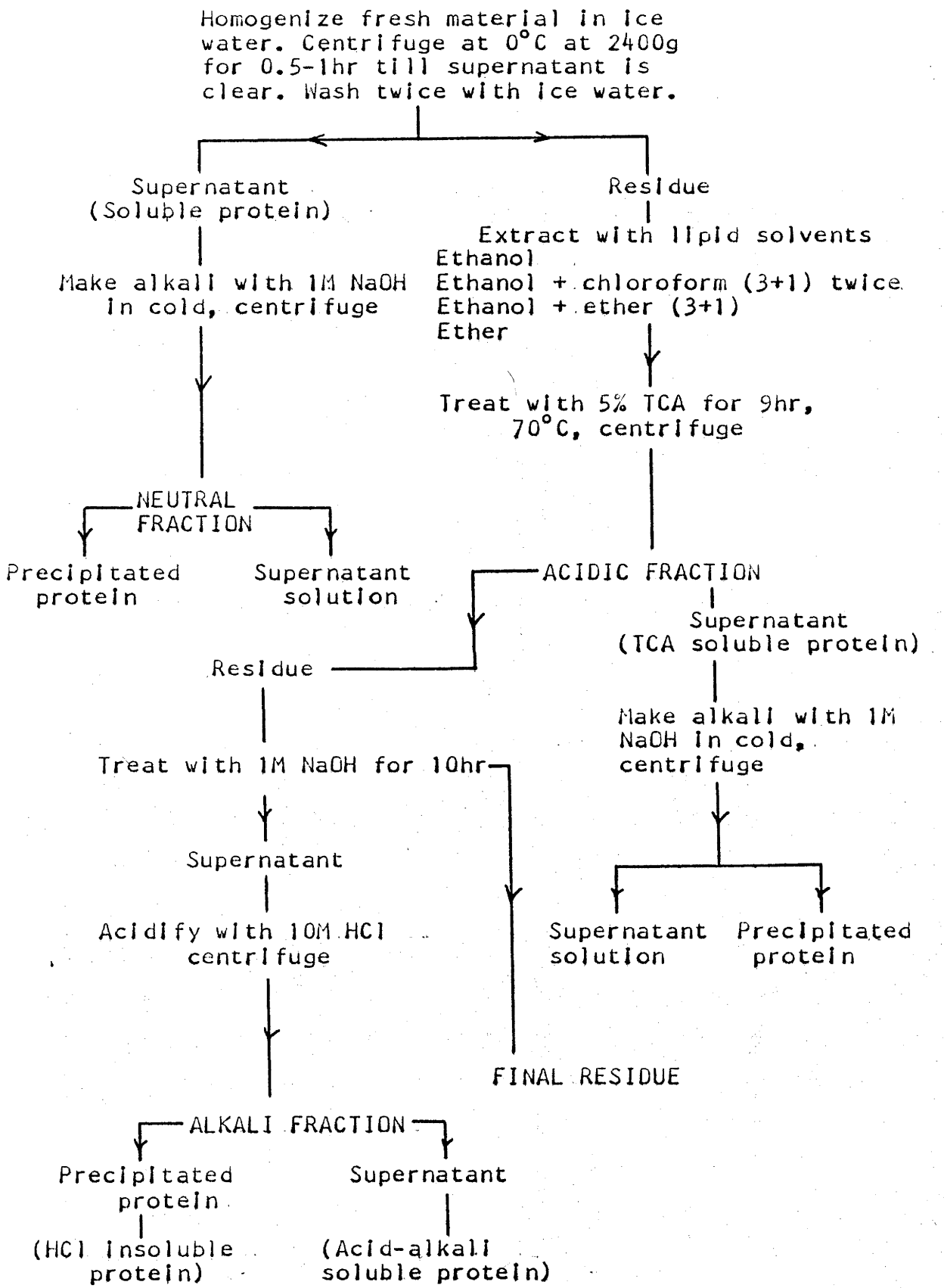
A standard technique to extract various protein fractions from P. radiata tissue was developed. Three primary fractions were extracted, a neutral, an acidic and an alkali fraction. Much of the protein in these fractions was then precipitated from them. A complete flow-chart of the system developed is presented in Fig 2 the derivation of the various steps involved is discussed below.

Neutral fraction

To attempt to produce the best possible homogenate in the initial breaking up of the fresh material, homogenates made with a detergent solution were tested. P. radiata needles from five month old seedlings were homogenized in an 0.05% solution of a commercial detergent, 'Pyroneg' (Diversey, A/asia Pty Ltd) with a 'Virtis' high speed homogenizer. The homogenate was centrifuged at 2400g for one hour and then resuspended twice in water, four times in 0.05% Pyroneg and finally in 1% NaCl. The NaCl was used to attempt to salt out further protein from the homogenate (Haurowitz 1963).

The first Pyroneg extract showed a very strong colour with the Lowry protein assay. The first water extract

Fig 2 Flow-chart of protein extraction procedures.



showed a very slight protein content and the second none. Subsequent Pyroneg extracts all showed appreciable protein content although much less than in the first extract. The final NaCl extract showed no detectable colour with the protein reagent.

The results suggested that repeated extraction with detergent continued to extract protein from the homogenate, whereas continued extraction with water did not. Extraction with 1% NaCl did not remove additional protein. Table 1 shows data for protein extracted from similar material by three different techniques to be discussed more fully later. In the Schmidt-Thannhauser extract, cold 10% tri-chloro acetic acid (TCA) was used instead of water for the initial homogenate. This yielded similar amounts of protein as were found with water extracts in the other two tests shown, but the protein quality in the extracts was not examined.

To standardize the procedure, water was selected as the better homogenizing solution than detergent. Although detergent homogenates were easier to prepare because of the detergent action, the continued removal of protein by the detergent with repeated extraction suggests that results for different extracts might not be comparable.

Use of lipid solvents

The use of lipid solvents in the extraction procedure is related to nucleic acid extraction rather than

protein fractionation and is discussed more fully in the nucleic acid section. With protein fractionation only, it probably had little value although it cleared the material of pigment. To standardize the procedure and allow for the instances when nucleic acids were also determined in the extracts, the lipid solvents were used in general routine protein fractionations.

Acidic fraction

Extraction with 5% TCA at 70°C has commonly been used in the Schneider nucleic acid procedure (Hutchinson and Munro 1961) which prompted its use here for acidic protein extraction.

Fig 3 shows the effect of different extraction periods on the yield of protein extracted with hot TCA in both root and shoot material. Seedlings used for this test were one month old. Soluble protein had been removed from the tissue by extraction with 0.1% Pyroneg. No lipid solvents were used. After 8-9 hours, maximum protein was extracted from both root and shoot tissue. For routine analysis thereafter, an extraction period of 9hr was used.

Alkali fraction

Insoluble protein can often be solubilized by heating with dilute NaOH (Haurowitz 1963). Ogur and Rosen (1950) used hot 2M NaOH to extract protein from the residue

after removal of nucleic acids from corn root tips. Hence, NaOH was used to extract alkali-soluble material from homogenates in this work.

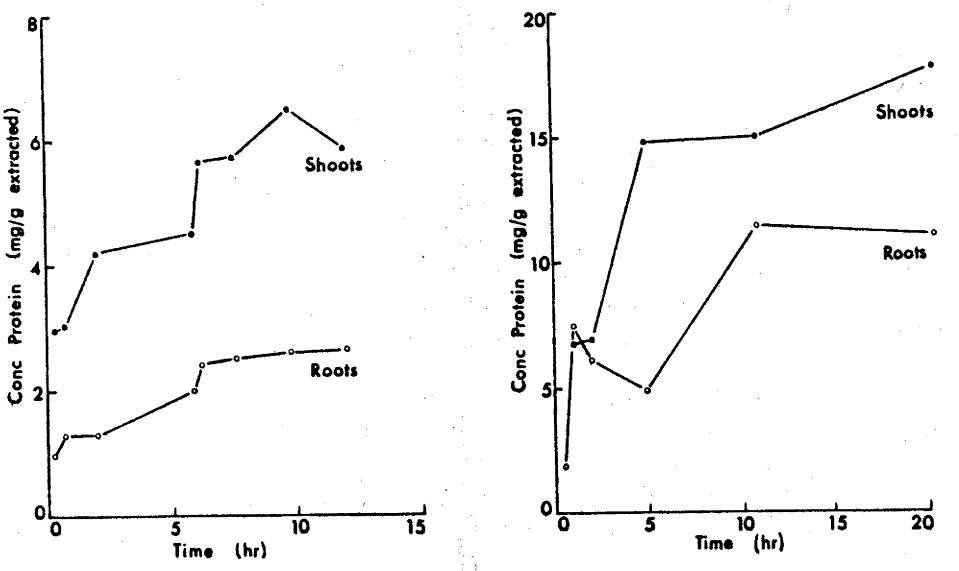


Fig 3 (Left) Effect of length of extraction period with 5% TCA at 70°C on protein yield (mg/g relative to fresh weight of material extracted) from homogenates of roots and shoots of one month old seedlings.

Fig 4 (Right) Effect of length of extraction period with 2M NaOH at 70°C on protein yield from residue left after complete acid extraction of Fig 3.

Fig 4 shows the effect of time on extraction of protein with 2M NaOH at 70°C from the residue left after extraction for 9 hours with 5% TCA at 70°C in the experiment above. In shoots there was a slight increase even with a 20hr extraction period. In roots, extraction was complete after 10hr.

For routine analysis, a 10hr extraction period was used. Later analyses suggested that 1M NaOH gave similar

results to a 2M solution: the more dilute alkali was subsequently preferred.

Precipitation of protein

Protein in solution can often be precipitated with the aid of various agents. Saturated or half-saturated solutions of $(NH_4)_2SO_4$ are often effective in 'salting-out' proteins. Alkali metal and Mg salts of sulphate and acetate are also used (Keller and Block 1960, Haurowitz 1963).

None of these techniques proved effective with any of the three extracts here. On standing for several days, especially in the cold, the solutions appeared cloudy. Acidifying the alkali fraction with HCl, or making the acid or neutral extracts alkali with NaOH caused rapid precipitation of dark brown material. The supernatants from the neutral and acidic extracts left after these solutions were spun down remained clear even after several weeks, but a slight cloudiness appeared in the alkali fraction after prolonged standing.

Table 1 shows the results of extractions using a modified Schneider method and the standard procedure of Fig 2. Needles from five month old seedlings were used for the extractions. The amounts of materials precipitated from the various solutions, measured by weighing after drying, are shown together with the protein levels in the solutions before and after precipitation as determined by the Lowry

Table 1 Concentration of protein (mg/g of fresh weight of extracted material) in various fractions extracted by different techniques. Each value is the mean of two samples.

Fraction	Schmidt-Thann-hauser	Schneider			Standard procedure (Fig 2)		
		Total	In soln. after pre-cipitation	Precip-itated	Total	In soln. after pre-cipitation	Precip-itated
Cold 10% TCA	32.1	-	-	-	-	-	-
Cold water	-	20.3	4.7	*	32.3	7.9	14.0
Cold 6% PCA	-	3.7	**	**	-	-	-
Hot 3%PCA 1hr 70 C	6.5	-	-	-	-	-	-
Hot 5%TCA 9hr 70 C	2.1	15.4	5.5	12.5	17.0	2.9	11.5
Hot 1M NaOH 20hr 70 C (Acid-alkali soluble)	17.4	24.3	19.2	5.7	26.3	21.8	0.1
HCl insoluble (precipit-ated from NaOH soluble extract with 10M HCl)	16.3	16.8	-	16.8	15.8	-	15.8
Total	74.4	80.5			91.4		

* Sample lost ** not precipitated - no sample

method. The alkali extract was separated into the precipitate and the supernatant because attempts were made to precipitate more material from the supernatant. The protein content of the acid-precipitated material was estimated by the Lowry procedure after it was redissolved in a small amount of 1M NaOH.

Only a small proportion of the total protein was left in the acidic and neutral supernatants after precipitation. More than one third of the protein in the alkali fraction was precipitated but much protein remained in the solution. On standing in the cold, very small amounts of material precipitated from this supernatant. In general, the sum of the weight of material precipitated and the weight of protein left in solution was very close to the weight of protein in the non-precipitated solution. This suggests that all the material precipitated from these fractions was protein.

Protein fraction nomenclature

The following nomenclature was adopted for the protein fractions. The protein extracted in the neutral and acidic fractions and subsequently precipitated from them was called soluble and TCA soluble protein respectively. The material precipitated from the alkali fraction was called HCl insoluble protein while the material remaining in the supernatant of the alkali fraction was called alkali-acid soluble protein.

Protein by Nesslerization

To test if measurement of N content of protein solutions by Nesslerization was possible, neutral, acidic and alkaline extracts of five month old P. radiata needles were prepared as outlined above. The solutions were treated with acid for colorimetric N determination as described earlier. Lowry protein content of the samples was also determined and converted to N content by dividing the result by 6.25.

Table 2 N concentration (mg/g relative to oven dry weight of original material extracted) of protein fractions measured by Nesslerization, Lowry or ninhydrin techniques (means of two replicates).

	Fraction		
	Neutral	Acidic	Alkali
Nesslerization	0.4	0.3	1.5
Lowry protein N	2.2	3.0	6.6
Ninhydrin amino-N	1.7	-	-

Amino-N concentration of the soluble fraction was determined with ninhydrin (Rosen 1957) against leucine standards and then converted to N content of the amino group. Results are shown in Table 2.

Even excluding the likelihood that other N compounds were present in the test solutions and not measured by the Lowry or ninhydrin techniques, the low N contents of the solutions determined by Nesslerization were striking.

The volatility of N compounds as the water in the solutions evaporated away during acid treatment probably led to the much reduced N content of these solutions.

Nesslerization appeared to be unsatisfactory for determination of protein content of solutions.

HEAVY METAL CONTENT OF PROTEIN FRACTIONS

To examine the relation between heavy metals and protein in P. radiata, a test was made of the Cu, Zn, Mn and Fe contents of the various protein fractions extracted.

Fresh needle material from five month old seedlings was fractionated according to the standard procedure of Fig 2. Solutions before and after precipitation and the precipitates themselves were oxidised with 1:7:24, H_2SO_4:$HClO_4$:HNO_3. Metal contents were determined with an atomic absorption spectrophotometer. Results are shown in Table 3.

For the soluble and TCA soluble protein, virtually all the metal was found in the material precipitated from the initial extracts and very little was left in the solution after precipitation. Consistently, the amount of metal in the precipitates exceeded the amounts in the solution before or after precipitation. This may have been due to loss of metal carried away with other vapours as the solutions dried out during acid treatment.

Table 3 Heavy metal content of protein fractions extracted according to the procedure of Fig 2. Metals expressed as ppm of the fresh weight of the needle material extracted. Each result is the mean of two samples.

Protein fraction		Cu	Zn	Mn	Fe
Soluble	Before pptn	0.8	17.1	32.6	64.5
	After pptn	0.0	1.1	0.6	1.1
	Precipitate	0.4	16.0	34.8	115.9
TCA soluble	Before pptn	2.9	8.2	2.4	36.8
	After pptn	0.6	1.7	0.5	6.5
	Precipitate	3.8	12.3	4.0	55.4
Alkali-acid sol	After pptn	1.8	2.8	1.5	73.0
HCl insoluble	Precipitate	0.7	0.1	0.0	0.9

For the alkali-acid soluble and HCl insoluble protein fractions, very little metal was precipitated from solution and most stayed in the alkali-acid soluble fraction. It was impossible to say if this metal was associated with the protein in the alkali-acid soluble fraction or with other components of the solution.

EXTRACTION AND ASSAY OF NUCLEIC ACIDS

Techniques tested

Fractions extracted from needles of five month old P. radiata seedlings by modified Schmidt-Thannhauser and Schneider procedures and the standard procedure of Fig 2 were tested for nucleic acid content. The fractions extracted are

shown in Table 4. The steps involved in the Schmidt-Thannhauser and Schneider extractions were derived as follows.

After initial extraction with cold TCA or water, the lipid solvents of Fig 2 were used. Hutchinson and Munro (1961) suggested that older procedures for nucleic acid estimation relied on determination of P content of tissue: this required that phospholipids be removed with organic solvents. With ultra-violet light nucleic acid assay, or chemical methods that do not rely on P determination, lipid removal may not be necessary. However, the unknown effects of pigments and nucleotide-amino acid complexes, which are removed by these solvents, on nucleic acid assays suggested that it might be wise to persist with the organic solvents.

To attempt to remove RNA from the tissue separately from DNA, cold 6% perchloric acid (PCA) extraction (Ogur and Rosen 1950) was tested in the modified Schneider procedure. Hot 3% PCA was used to attempt to remove both RNA and DNA in the modified Schmidt-Thannhauser procedure. Neither of these procedures removed any material reacting with diphenylamine (Table 4). For the hot PCA this was most unusual as DNA is normally extracted by hot acid in the standard Schneider procedure.

From this point in the procedures the conventional steps (Hutchinson and Munro 1961) in Schmidt-Thannhauser and Schneider procedures were followed (Table 4). After the 1hr alkali extract of Schmidt-Thannhauser followed a hot TCA then

Table 4 Concentration of orcinol reacting material (mg/g) and diphenylamine reacting material (µg/g) relative to fresh weight of extracted material in various extracted fractions, against RNA and DNA standards respectively. Each value is the mean of two samples.

Fraction	Orcinol reacting material (mg/g)					DPA reacting material (ug/g)				
	ST	Schneider		Standard *		ST	Schneider		Standard *	
		Total	After pptn	Total	After pptn		Total	After pptn	Total	After pptn
Cold 10% TCA	14.8	-	-	-	-	0	-	-	-	-
Cold water	-	11.1	6.9	10.7	5.2	-	0	55.8	0	98
Cold 6% PCA, -10 C, 21hr	-	1.4	**	-	-	-	0	**	-	-
Hot 3% PCA, 70 C, 1hr	24.6	-	-	-	-	0	-	-	-	-
0.3M NaOH, 40 C, 1hr Alkali-acid soluble	7.4	-	-	-	-	54.5	-	-	-	-
HCl insoluble	4.7	-	-	-	-	97.5	-	-	-	-
Hot 5% TCA, 70 C, 9hr	10.3	45.6	27.2	33.3	14.2	0	0	17.6	0	154
1M NaOH, 70 C, 20hr Alkali-acid soluble	9.6	14.8	9.5	11.7	9.4	116.5	91.5	30.0	148	176
HCl insoluble	1.4	4.3	-	-	-	79.0	256.5	-	-	-
Total	72.8					346.5	348.0			

DPA diphenylamine ST Schmidt-Thannhauser technique * Standard procedure of Fig 2.
** Not precipitated - No sample in this extract

another hot alkali extract to attempt to extract any residual nucleic acid. The hot TCA extract of the Schneider procedure was followed by a hot alkali extract to see if further material could be extracted. Material was precipitated from the Schneider and standard procedure fractions as described for protein assay. HCl insoluble material was redissolved in 1M NaOH for nucleic acid assay.

Results of nucleic acid colorimetric assays

The concentrations relative to the fresh weight of the extracted material, based on RNA and DNA standards, of substances reacting with orcinol or diphenylamine in the various fractions extracted are shown in Table 4. In the Schmidt-Thannhauser extract RNA would be expected in the hot PCA extract and, if not all extracted in that fraction, in the first alkali-acid soluble fraction. Normally (Hutchinson and Munro 1961), 1hr in 0.3M NaOH is long enough to extract all the RNA. But much orcinol reacting material was found in the hot TCA extract and the subsequent second hot alkali extract. In both the Schneider and standard procedures, the highest concentration of orcinol reacting material was found in the TCA extract. This fraction would normally be expected to contain RNA, so this result suggested that these fractions did contain RNA. The concentration of orcinol reacting material left in solution after precipitation was lower than before precipitation.

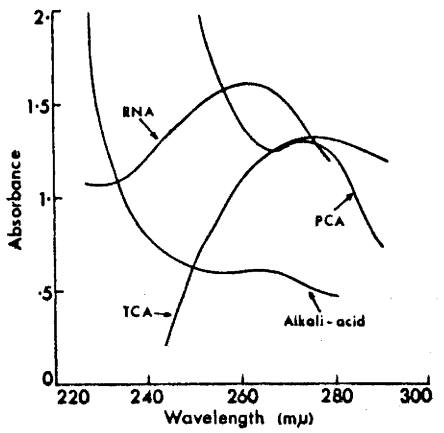
A number of substances other than RNA may react

with orcinol, particularly carbohydrates and large amounts of protein (Hutchinson and Munro 1961). The orcinol reacting material precipitated from solution was probably protein, since it was mainly protein that was precipitated from these solutions (see earlier). However, because interfering substances appeared in all fractions, the assay was unreliable and seemed unsuited for conventional extraction procedures with P. radiata tissue.

Diphenylamine reacting material was found only in solutions left after precipitation of material and in the HCl insoluble material which was redissolved in NaOH for assay. This suggested that precipitation released substances reactive with diphenylamine. Sugars and protein both react with diphenylamine (Hutchinson and Munro 1961) and these may have been involved here. The standard procedure for DNA extraction and assay seemed quite unsuited to P. radiata tissue.

Ultra-violet light spectrophotometric assay of nucleic acids

Fig 5 shows scans of several of the solutions extracted in the Schmidt-Thannhauser procedure compared with a pure sample of RNA. Fig 6 shows scans of solutions left after precipitation of material from the fractions extracted by the Schneider procedure compared with a scan of pure DNA.



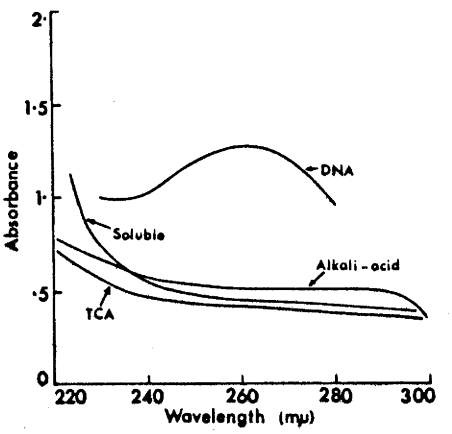


Fig 5 (Left) and Fig 6 (Right) Ultra-violet light spectra of solutions extracted by the Schmidt-Thannhauser procedure compared with a 40μg/ml sample of pure RNA (Fig 5) and by the Schneider procedure compared with a 40μg/ml sample of pure DNA (Fig 6). In both cases, background absorption due to TCA has been subtracted from the scans for the TCA soluble fractions.

In none of the solutions was there a peak near the maxima for RNA or DNA. The maxima at around 270mμ for the extracts in Fig 5 correspond to those for polypeptides (Fleck and Munro 1962, Munns and Johnson 1960). This suggested protein in the solutions interfered with nucleic acid assay, if any nucleic acid was present. These peaks were not present in solutions from which material had been precipitated (Fig 6). This supports the earlier finding that the precipitated material was protein. The alkali-acid soluble fraction contained much protein both before and after attempts were made to precipitate material from it (Table 1). The UV scans in Figs 5 and 6 showed little evidence that these solutions contained protein with similar UV characteristics to those in other fractions.

SUMMARY AND CONCLUSIONS

Fractionation of P. radiata tissue at neutral, acid and alkali pH produced recognisable protein containing fractions. Almost all the protein could be precipitated from these fractions, except protein soluble in acid and alkali remained in solution in one of the fractions. It seemed likely that all the material precipitated from the neutral and acidic fractions was protein. Addition of alkali to neutral or acidic extracts or acid to alkali extracts caused precipitation, especially in the cold. Salting out or coagulation of protein by heating were unsuccessful in causing protein precipitation.

Most of the heavy metals Cu, Zn, Mn and Fe found in the neutral and acidic fractions was precipitated with the protein on addition of alkali. Very little metal precipitated with the HCl insoluble material but was retained in the alkali-acid soluble fraction.

Loss of N or heavy metals by vapourisation during acid digestion of solutions made protein estimates by Nesslerization and estimates of metal contents unreliable for materials in solution.

It was not clear if nucleic acids were extracted from P. radiata tissue with the protein fractions. Interference, possibly by protein and carbohydrate, with the orcinol and diphenylamine reactions for RNA and DNA respectively made it impossible to determine in which frac-

tions nucleic acids were extracted, if at all. Ultra-violet light spectrophotometry produced no evidence of nucleic acids in these extracts either before or after precipitation. Conventional Schmidt-Thannhauser and Schneider techniques seemed quite unsuited to *P. radiata* tissue.

PAPER 4

RELEASE OF STORED METAL, AND UPTAKE AND TRANSPORT OF HEAVY METALS IN SEEDLINGS OF *PINUS RADIATA* D.DON

ABSTRACT

Both Cu and Zn which had been stored in roots of *P. radiata* seedlings during pretreatment with high metal levels were rapidly released to shoots when seedlings were placed in deficient solutions for three months. This release may have involved storage protein turnover. The correlation between the rates of uptake by roots and transfer to shoots of Cu, Zn, Mn and Fe were studied. These metals appeared to have different root uptake mechanisms, but the same mechanism for release to the xylem stream when all were in adequate supply.

Cu or Zn deficiency reduced transpiration rate in these seedlings. However, rate of transport of these metals to shoots was not correlated with transpiration rate: this may be due to the recently postulated mechanism for metal transport in the xylem. The long term nature of the experiment cast doubts on the validity of some of these conclusions.

INTRODUCTION

Previous work in this series suggested that when an excess of heavy metals (Cu, Zn, Mn or Fe) was available to *P. radiata* seedlings in nutrient solutions, the excess was stored in roots in association with protein. If held at protein surfaces, metals are generally readily released from the protein by treatment with dilute acids (Haurowitz 1963). But in subsequent work in this series, substantial amounts of metals were found to be associated with protein precipitated after treatment with acids. Very little metal was left in the supernatant after precipitation. This suggests that the excess metal stored in association with protein is firmly bound within the protein structure, perhaps during secondary or tertiary folding (Li 1966). This may prevent release of the stored metal if the plant should encounter metal stress conditions.

The experiment reported here was primarily designed to determine whether or not stored metal was released by roots when the seedling was exposed to metal deficiency.

If metals stored in roots are available for release to shoots, then their ultimate availability will depend on the control mechanisms which affect their transfer to the shoot and also, in the long run, their uptake by roots. The literature relating to the control of ion uptake by roots and transport to shoots is briefly reviewed here so that subsequent discussion on metal transport in seedlings may be put in perspective.

Work on ion uptake by roots and release to the xylem stream has largely been restricted to short term studies with Cl^- and alkali metals, especially K^+ (Dunlop 1974, Latles 1969, Lauchli 1972). For these ions, uptake into the cell is controlled by dual, active mechanisms (Epstein 1966, Latles 1969) and debate is continuing as to where in the cell and how these mechanisms operate (Lauchli 1972, Latles 1969, Nissen 1973a,b). The first mechanism works at low external ion concentrations ($5x10^{-4}$ - 10^{-3}M) and is specific for a particular ion. The second is less specific and operates on a number of ions when they are present at higher external concentrations.

After active uptake at the root epidermis and cortex, nutrient ions move passively in association with water across the root tissue to the stele. This transport may occur within the cell walls or through the cells themselves via plasmodesmatal connections (Lauchli 1972, Molz and Ikenberry 1974). The latter pathway seems to be more important (Milthorpe and Moorley 1969). The Casparian strip and, in some cases, secondarily thickened endodermis are thought to prevent any passive flow of those ions which move within the cell wall into the xylem. Instead, these ions are forced to pass into living cells which may then actively control their fate. There may occasionally be leaks in the endodermal barrier in some plants, but these aberrations are thought to have only slight overall effects (Kramer 1969, Lauchli 1972, Brouwer 1965). For the most part, ions are actively secreted into the xylem vessels (Lauchli 1972,

Laties 1969, Lauchli et al 1971) although the direction of the active transport has been questioned (Dunlop 1974). The xylem parenchyma cells seem to be the site of active release (Lauchli et al 1971, 1974).

After release to the xylem, ions move in association with the transpiration stream, sometimes very rapidly (up to 60m/hr in rapidly transpiring trees) (Kramer 1969). A very close correlation has been observed between transpiration rate and the rate of ion movement to shoots. Brouwer (1965) suggests this occurs because changes in the rate of centripetal water flow across the root actually affect the rate of the active process which releases ions to the xylem stream. A less recent review (Scott Russell and Barber 1960) maintains that when the plant is under nutrient stress, active control of ion release to the xylem is tighter and ion movement does not necessarily correlate with transpiration rate.

In addition to examining release of stored metals (Cu, Zn, Mn and Fe) from roots to shoots, the experiment reported here also examined the dynamics of uptake of these metals by roots and release to shoots both in relation to each other and in relation to the transpiration stream. This work differed substantially from other work on ion transport in that it was a long term (three month) study.

MATERIALS AND METHODS

Experimental

Three-week-old *P. radiata* seedlings were transferred to plastic pots containing nutrient solutions and grown in the glasshouse as described for earlier experiments. When Zn and Cu were present in normal concentrations in nutrient solutions, they were both supplied at 0.1ppm which has been shown earlier to be adequate for both elements.

Seedlings were grown for 10 weeks with one of four nutrient pretreatments, (a) no Zn, (b) Zn at 1.0ppm, (c) no Cu or (d) Cu at 1.0ppm. After 10 weeks, treatments (b) and (d) were stopped and those seedlings subsequently received solutions with no added Zn or Cu respectively. At the same time, half the seedlings were covered with shading material which gave 40% of full light intensity. Shading was applied to alter growth and transpiration rates to allow comparison of rates of metal movement in seedlings under different growth conditions. Seedlings were grown for another 12 weeks with these new treatments. A total of 16 pots of seedlings were used, two for each nutrient pretreatment - level of shading combination. When seedlings were harvested, samples from each pair of pots were pooled. Nutrient solutions were changed fortnightly.

Some seedlings which were pretreated with high Zn levels were killed by a damping-off fungus (*Pythium* sp), thought to be promoted by high Zn levels (Heather, pers. comm.). The effect was more pronounced with shaded

seedlings although it decreased after transfer to Zn deficient solutions. Growth of the surviving seedlings was somewhat checked during the infection period. As a result, many data for seedlings pretreated with 1.0ppm Zn and subsequently grown in 40% of full light were missing. Generally results from this treatment were not included in the analyses.

Measurements

Seedlings were harvested 0, 4, 8 and 12 weeks after the high Zn or Cu pretreatments were stopped. Seedlings were separated into shoots and roots, weighed fresh, dried at 85°C and reweighed. Levels of Cu, Zn, Mn and Fe in the dry plant material were determined by atomic absorption spectrophotometry as described in previous experiments.

Pots were weighed weekly to determine water loss by transpiration and hence the amount of water passing through each seedling over each measurement period. Tests showed there was negligible water loss from pots with no seedlings.

Temperature and relative humidity were monitored continuously with a thermohydrograph. Areas under the thermohydrograph curves were estimated to find values for degree-days and relative-humidity days during each period between harvests.

RESULTS

Effects on growth

Figures 1 and 2 show the relationship between seedling shoot and root fresh weight and time during the measurement period. A logarithmic conversion converted the growth curves to straight lines. The slopes of the regression lines, the proportion of the variance in $\log_e$ fresh weight explained by each regression and the covariance adjusted mean fresh weights for each regression line are also shown. The results were identical for fresh weight and oven dry weight: over all the data, fresh weight correlated 0.99 with oven dry weight in shoots and 0.98 in roots. Discussion is therefore restricted to fresh weight (arbitrarily).

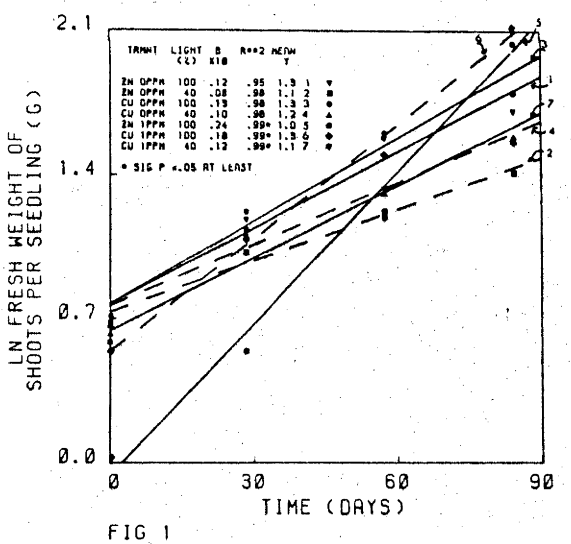


FIG 1

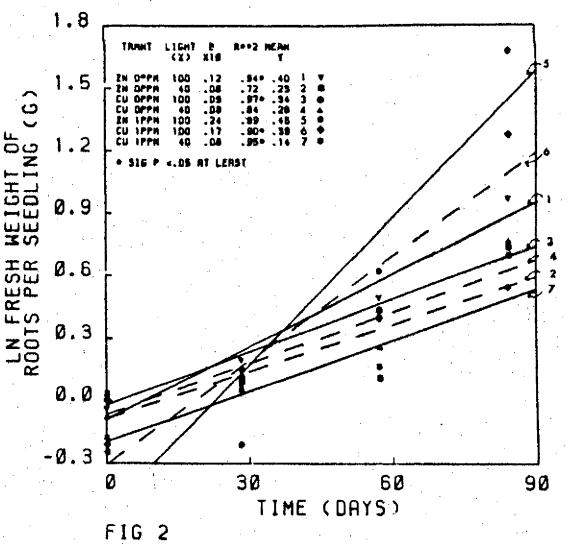


FIG 2

Figs 1 and 2 Growth of seedlings which received different treatments, as a function of time. B = regression coefficient, R**2 = the proportion of the variance explained by regression, mean Y is shown covariance adjusted.

Covariance analysis of the results in Figs 1 and 2 showed there were significant differences between slopes of these regression lines in shoots ($p<.001$) and roots ($p<.05$). The simultaneous test procedure (Sokal and Rohlf 1969) with $p=.05$ showed that in shoots the slopes of the regression lines pretreated with high Zn or Cu and grown in full light were significantly greater than for other pretreatments. Slopes of these two lines did not differ significantly, nor did slopes of the remainder. In roots, a similar effect applied, except that the slope of the line from seedlings pretreated with high Cu and grown in full light was intermediate and did not differ significantly rom the other two sets of lines.

These results suggest that seedlings pretreated with high Zn or Cu levels and grown in full light showed faster shoot and root growth rates than those receiving other treatments. As will be discussed later (Figs 3-6), the Zn and Cu nutrient status of these seedlings was much better than that of seedlings pretreated with low Zn or Cu, at least for part of the growth period: this explains their higher growth rates. The seedlings pretreated with high Cu, but grown in 40% light, showed a lower growth rate than those pretreated with high Cu and grown in full light. This suggests that light had become limiting to growth with only 40% of full light irrespective of seedling nutritional status. A test of the significance of the differences between covariance adjusted mean shoot weights of seedlings pretreated with high Zn or Cu and grown in full light, showed

that seedlings pretreated with high Cu had a significantly ($p<.025$) greater mean shoot weight than those pretreated with high Zn. This was probably due to the effects of fungal attack which reduced growth of seedlings pretreated with high Zn levels (see earlier). Similar effects did not apply in roots.

For the remaining treatments, for which slopes of growth curves did not differ significantly, a test of the significance of the differences between covariance adjusted mean fresh weights showed there was a significant ($p<.025$ at least) increase in shoot and root fresh weight with full light when compared to 40% full light, but no significant effects of pretreatments. These results are summarized in Table 1.

The conceptual interpretation of the effects of light is difficult to grasp. The logarithmic conversion of data attempts to convert growth curves to straight lines. Because seedlings grown in full and 40% light had the same initial weights at the commencement of the growth period, one would expect a difference in slopes of logarithmic growth curves if there were effects of light on growth rates. If there were differences in slopes, then it would be meaningless, of course, to test adjusted means of the dependent variable ($\log_e$ fresh weight in this case).

Table 1 Effects of light intensity and seedling pretreatment on fresh weight of shoots and roots of seedlings other than those pretreated with high Cu or Zn levels and grown in the light. Data shown as mean $\log_e$ fresh weight (g) adjusted by covariance with time. Slopes of regression lines did not differ significantly (Figs 1,2)

(a) Effects of pretreatment

	Pretreatment			Signif-icance (p<)
	Zn 0ppm	Cu 0ppm	Cu* 1ppm	
Shoots	1.18	1.26	1.14	NS
Roots	.31	.31	.14	NS

* Data for seedlings in 40% light only - this explains the apparently lower growth with this pretreatment.

(b) Effects of light intensity

	Light (%)		Signif-icance (p<)
	100	40	
Shoots	1.31	.37	.025
Roots	1.13	.22	.025

It seems likely here, that the data were insufficient and the differences not large enough to recognize differences between slopes of regression lines, but it was possible to detect differences in adjusted means. As well, the logarithmic conversion may not have produced as good a straight line fit as desired. Study of the regression coefficients in Figs 1 and 2 shows that seedlings grown in full light consistently had higher coefficients than those grown in 40% light. This suggests the test of adjusted means reflects real differences in growth rates of seedlings in full and 40% light.

Effects of treatments on metal transfer to shoots and uptake by roots

As shown in the previous section, different treatments had different effects on seedling growth rates. Such differences in growth rate might be expected to affect rates of metal movement to shoots and uptake by roots independent of any effect of the treatments themselves. As well it seemed most likely that the rate of increase of plant weight over any time period would be most likely to affect rates of nutrient change, because nutrients would move to shoots or be taken up by roots in response to the new growth in that period. So, to examine effects of treatments on rates of metal movements to shoots or uptake by roots, the rate of metal change during each of the three measurement periods was expressed per unit rate of change of fresh weight in the same period.

The effects of seedling pretreatment and light intensity on rate of metal change per unit rate of change in fresh weight are summarised in Table 2. For roots, the data represent the total amount of each metal passing into the root during the measurement period, i.e. the total amount taken up by the seedling. For shoots, the data represent only the amount of each metal passing into the shoot. Because the data were incomplete the interactions between the factors were not examined. As indicated in Table 2, the variances in many samples were heteroscedastic. Attempts to make variances homogenous with transformations were

Table 2 Main effects in variance analysis of pretreatments and light intensity on rates of metal change (µg metal per seedling per day) per unit rate of change of fresh weight (g per seedling per day). For both sets of analyses, the data were balanced over each treatment, i.e. data for Zn pretreatments included data only for seedlings grown in full light, because data were missing where seedlings were grown with high Zn levels in 40% of full light.

(a)Effects of pretreatments

		Pretreatment				Signif-icance (p<)
		Zn 0ppm	Zn 1ppm	Cu 0ppm	Cu 1ppm	
Shoots	Cu	1.22a	1.22a	.24b	.68ab	.01*,**
	Zn	2.4a	3.4a	12.0b	11.0b	.025**
	Mn	66.0	73.1	88.6	82.1	NS
	Fe	27.5	44.9	42.8	32.9	NS
Roots	Cu	4.99a	2.63a	.84b	.61b	.10*,**
	Zn	8.3	10.2	92.4	53.8	NS*
	Mn	203.	126.	369.	403.	NS
	Fe	251.	216.	880.	299.	NS

(b)Effects of light intensity

		Light (%)		Signif-icance (p<)
		100	40	
Shoots	Cu	.41	.77	NS*
	Zn	6.9	11.2	NS*
	Mn	66.1	96.7	NS*
	Fe	24.9	45.4	NS*
Roots	Cu	1.42	2.64	NS*
	Zn	33.6	77.0	NS*
	Mn	249.	472.	NS*
	Fe	301.	724.	NS*

* Variances were not homogeneous in different samples.
** Means with similar letters alongside did not differ significantly (t test with p=.05)

unsuccessful. The tests for homogeneity of variance and subsequent test for equality of means given by Sokal and Rohlf (1969) were used where appropriate: otherwise, normal variance analysis was used.

Seedlings pretreated with low or high Zn, which subsequently received deficient Zn levels during the measurement period, showed much lower rates of Zn uptake by roots and movement to shoots than those receiving adequate Zn throughout the experiment (i.e. seedlings pretreated with low or high Cu levels) (Table 2a). Although the effect in roots was non-significant, the means were so different it seems likely the non-significance represents Type II error. Similar effects were observed with Cu for seedlings pretreated with low or high Cu. These results might reasonably be expected.

Although the effects were not significant, it appears that seedlings pretreated with high Zn or high Cu showed faster rates of movement of Zn or Cu respectively to shoots than seedlings pretreated with corresponding low metal levels (Table 2a). This suggests there may have been release of excess metal stored in roots to shoots when seedlings were placed in deficient solutions. To examine this more closely, Figs 3-6 show the actual amounts of Zn or Cu found in seedlings with different pretreatments. The metal contents are plotted against seedling fresh weight so the metal uptake - rate of growth relationship can be judged. The metal concentrations relative to oven dry weight in shoots or

Figs 3-6 Actual amounts of Cu (Figs 3,4) or Zn (Figs 5,6) found in seedlings with different treatments and measured at different harvests, but drawn relative to seedling fresh weight at each harvest. Concentrations (ppm relative to oven dry weight) of Cu or Zn respectively in the seedlings at the time of harvest are shown alongside each observation.

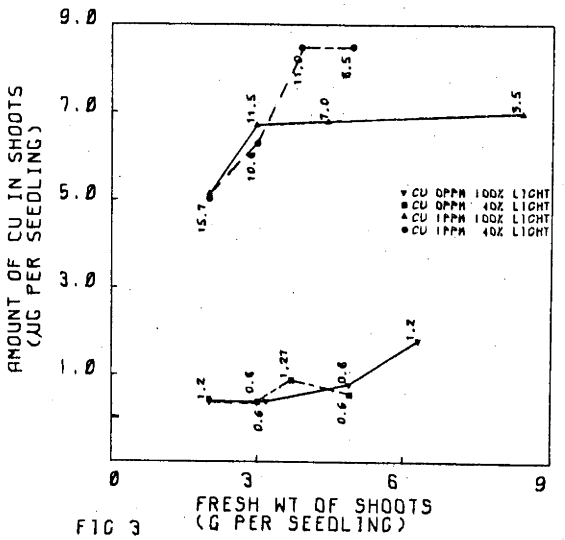


FIG 3

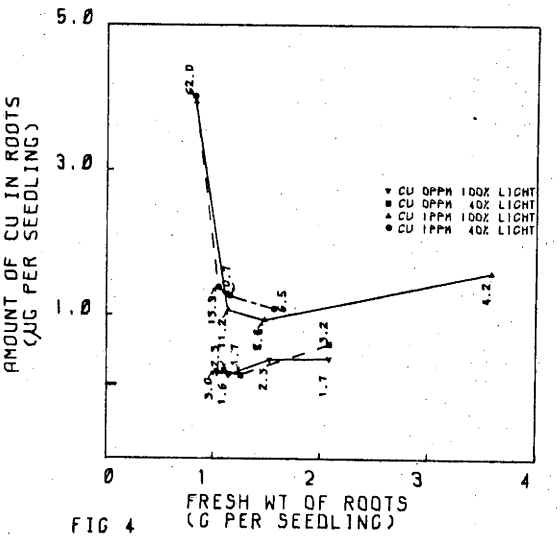


FIG 4

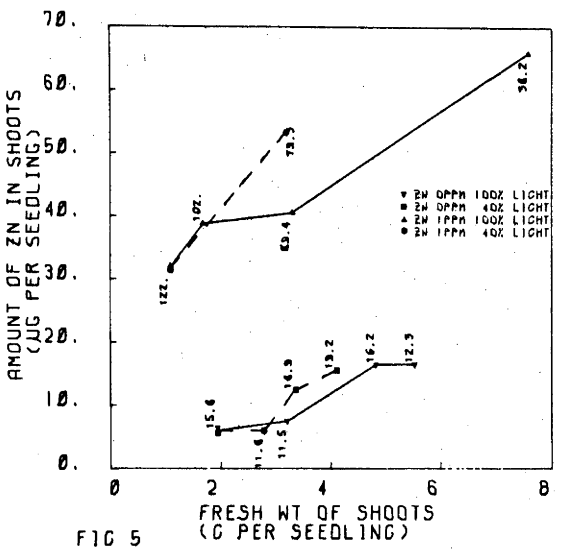


FIG 5

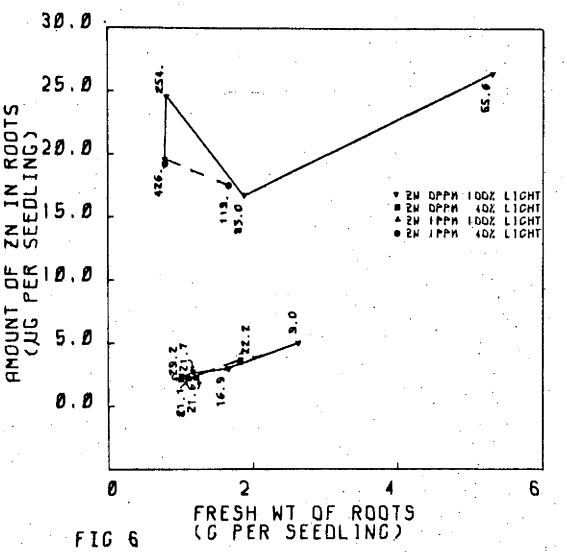


FIG 6

roots are shown alongside each observation. There was, of course, a direct correspondence between growth and time for each treatment shown in these Figures. The concentrations of metals at the time of the first harvest show that pretreatment with high Zn or Cu gave very high metal concentrations, especially in roots, consistent with findings in previous experiments.

For Cu (Figs 3,4), the trends seem quite clear. Seedlings pretreated with low Cu levels showed only slight increases in amounts of Cu per seedling in shoots and roots over the whole growth period as seedling size increased with time. Cu concentration in both shoots and roots remained well below sufficiency (6ppm in shoots and 20ppm in roots as found in earlier work) throughout the growth period. The fact that there was some uptake of Cu as seedlings grew shows there was some residual Cu left in the nutrient solutions which had not been removed by purification of nutrient salts.

In roots of seedlings pretreated with high Cu levels, there was initially a very marked fall in the amount of Cu per seedling (Fig 4) as seedlings grew with time. By the time of the first harvest seedlings had about 12ppm Cu in roots which is well below sufficiency. Thereafter, there was a slight further decline and eventually a slight increase in unshaded trees in total amount of Cu in roots. Concentrations of Cu in roots continued to decline till they were almost as low as those in trees with low Cu pretreatment at the final harvest.

In shoots, there was initially a very sharp rise in Cu content of seedlings pretreated with high Cu, but as the seedlings grew further, there was virtually no further increase (Fig 3). Concentration in shoots declined to below sufficiency levels soon after the second harvest in seedlings grown in full light, but had not quite reached deficiency by the final harvest in the slower grown seedlings in 40% light.

These results are consistent with a rapid release of the Cu stored in roots during high Cu pretreatment after the seedlings were placed in deficient solutions. The rapid fall in concentration in roots to below sufficiency levels by the first harvest suggested that all the stored Cu had been released by that time. This explains the sudden sharp rise in Cu content of shoots but with little subsequent change in Cu content at later harvests.

For Zn, the effects were not quite as marked. Seedlings pretreated with low Zn showed showed small increases in Zn contents of shoots and roots (Figs 5,6) and fairly static, deficient Zn concentrations (deficiency with less than 30ppm Zn in shoots and 70ppm in roots).

In roots of seedlings pretreated with high Zn levels there was a rapid decline in Zn concentration in roots as seedlings grew. However, only the faster growing sedlings in full light reached deficiency levels (55.6ppm Zn) and then only at the last harvest (Fig 6). In shoots of seedlings pretreated with high Zn levels there was also a decline in Zn concentration with time, but even by the final harvest the

fastest growing seedlings in the light still had a Zn concentration just sufficient for unimpaired growth (38ppm) (Fig 5). Had there been no Zn released from roots or taken up from solution during the measurement period, Zn concentration in shoots of the fastest grown seedlings would have declined to 18ppm, well below sufficiency, if they had grown to the same final size that they did finally achieve. This suggests conditions were such that release of excess Zn from roots for transfer to shoots might have been expected. The rather more gradual decline in concentration to deficiency in these seedlings when compared to the decline in Cu concentration in seedlings pretreated with high Cu levels, suggests these seedlings did not suffer as great a stress as the Cu deficient seedlings. Hence, if there was release of stored Zn to shoots, it would have occurred at a much more leisurely pace than was the case with Cu and would have become confused with Zn taken up from the nutrient solution. This suggests there was relatively more Zn available in the Zn deficient solutions than Cu in the Cu deficient solutions. The results given in Table 2a do, however, suggest that seedlings pretreated with high Zn showed higher rates of Zn movement to shoots than those pretreated with low Zn.

Generally then, it appears there was release to the shoots of excess Cu or Zn stored in roots during pretreatment with high metals when seedlings were placed in deficient solutions.

There were no significant effects of pretreatment

method on rate of Mn or Fe movement to shoots or uptake by roots (Table 2a). Nor were there significant effects of shading on rates of movement of any metal to shoots or uptake by roots (Table 2b) as assessed by the t test for samples which display heteroscedasticity (Sokal and Rohlf 1969). However, in all cases the mean rate of metal uptake by roots or movement to shoots in seedlings grown in 40% full light exceeded that in seedlings grown in full light. Sample variances from the former exceeded those from the latter. Assuming the populations from which the samples were drawn had a normal distribution, and that the population means were the same, then at most one might expect 50% of samples from one treatment to have, by chance, means greater than those from the other treatment. Because all the sample means from sedlings grown in 40% full light exceeded those from seedlings grown in full light, it seems likely that the population means for the former did in fact exceed those for the latter. The non-significant t tests are probably due to the conservatism built into these tests to allow for the assumptions made in deriving the techniques. A higher rate of nutrient uptake or transport per unit rate of change of fresh weight for seedlings in 40% full light probably represents an effect of their overall slower growth rate, but maintenance of the same rate of metal uptake.

Intercorrelations amongst rates of metal movement to shoots and uptake by roots.

Substantial differences between rates of uptake of metals by roots and transport to shoots per unit rate of change of fresh weight were observed in seedlings receiving different nutrient treatments. The coefficients of variation (standard deviation of a mean divided by the mean - a measure of relative variability in a set of data) of the data over all treatments varied from at least 45% to at most 244% for different metals (Table 3). Large variations like these were expected when the experiment was established because of differences in availability of nutrients to seedlings, especially seedlings pretreated with high Zn or Cu levels. This may have been the cause of the variation in the data for Zn, which was released from storage under deficiency conditions more slowly than excess Cu which was all released to shoots within the first four week measurement period (discussed earlier). However, for Cu, Mn and Fe, the large variations observed in rates of metal uptake and movement were most probably due to the substantial differences in physiological age caused by the different effects of treatments on growth rates (see earlier).

Because the variations were large, they provided a reasonable spread of data to try to determine if the movements of these metals were related to each other. However, because seedlings at different stages of growth may have different nutrient requirements, the results must be

interpreted cautiously.

Figs 7 and 8 show the relation between the rates at which the various metals moved to shoots per unit rate of change of fresh weight. For the four metals assayed, there are six such figures which could be drawn, but the two shown are sufficient to explain the rationale for the subsequent analyses.

Table 3 Coefficients of variation of rates of metal uptake by roots or transport to shoots (μg metal per seedling per day per unit rate of change of fresh weight). Data were combined from all treatments in the experiment.

	Coefficients of variation (%)	
	Shoots	Roots
Cu	137	244
Zn	80	143
Mn	45	69
Fe	87	101

The Figures firstly show that for Cu there were negative rates of movement to shoots over some time periods. This suggests that the measures of metal movement taken were only net effects and that there was movement of metals from shoots to roots as well as from roots to shoots. The factors which control transport from shoots to roots may be quite different to those that control transport from roots to shoots. The subsequent results are interpreted with this in mind.

Figs 7-12 Relationships between rates of uptake by roots or transport to shoots for different metals. Metal rates expressed as µg metal per seedling per day per unit rate of change of fresh weight.

Data separated into two populations: seedlings receiving low levels of Zn, (Low Zn), (i.e. pretreated with low or high Zn) or low Cu, (Low Cu), (i.e. pretreated with low or high Cu). Correlation coefficients (r) for the individual populations or the combined data are shown as appropriate. Sts = Shoots, Rts = Roots.

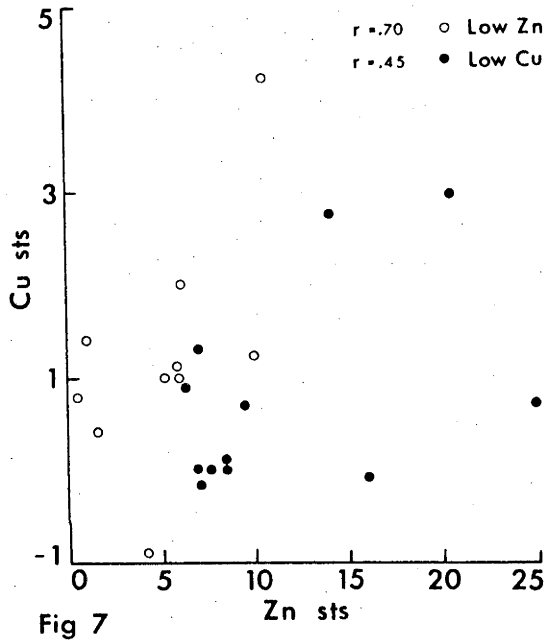


Fig 7

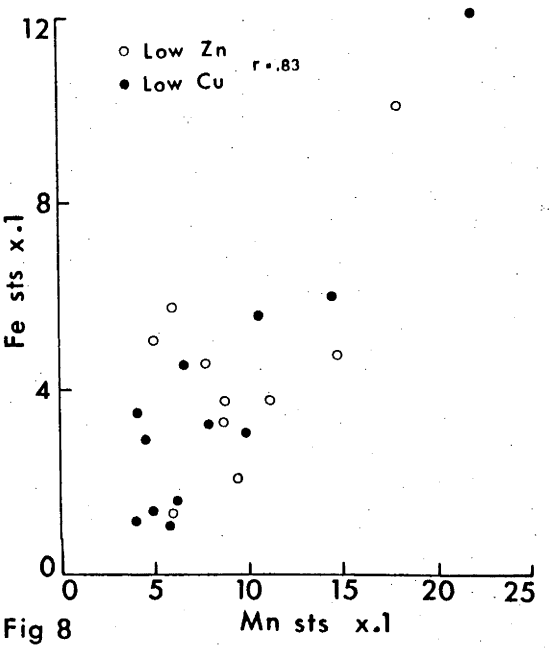


Fig 8

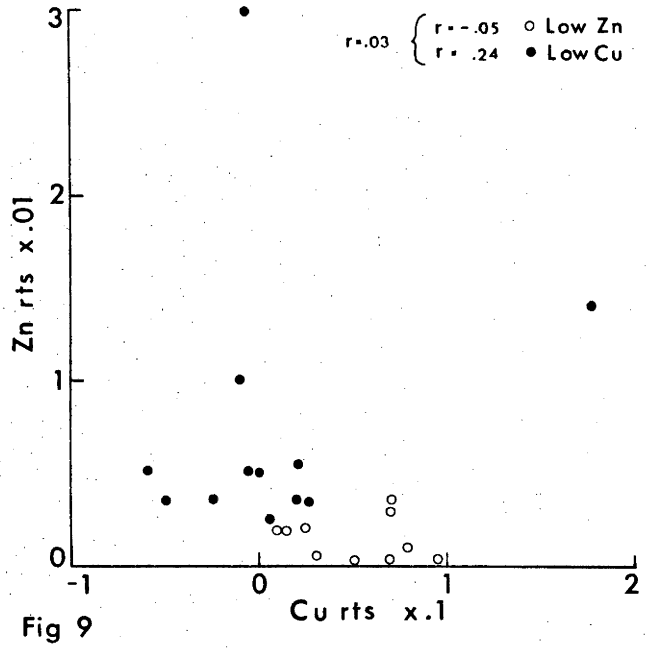


Fig 9

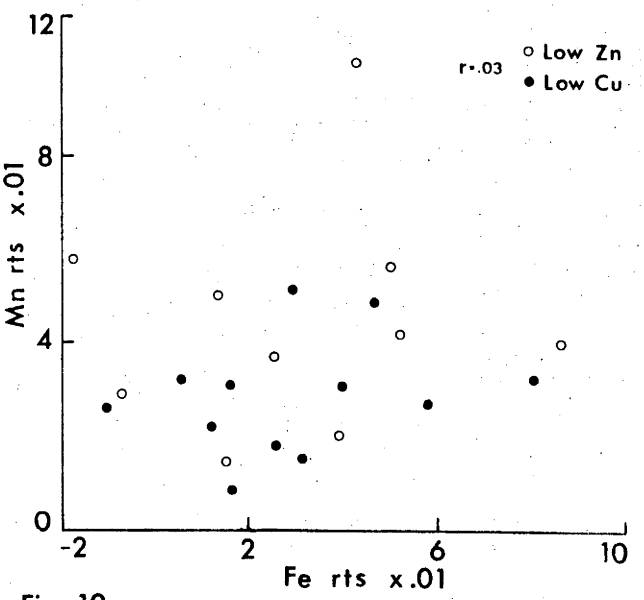


Fig 10

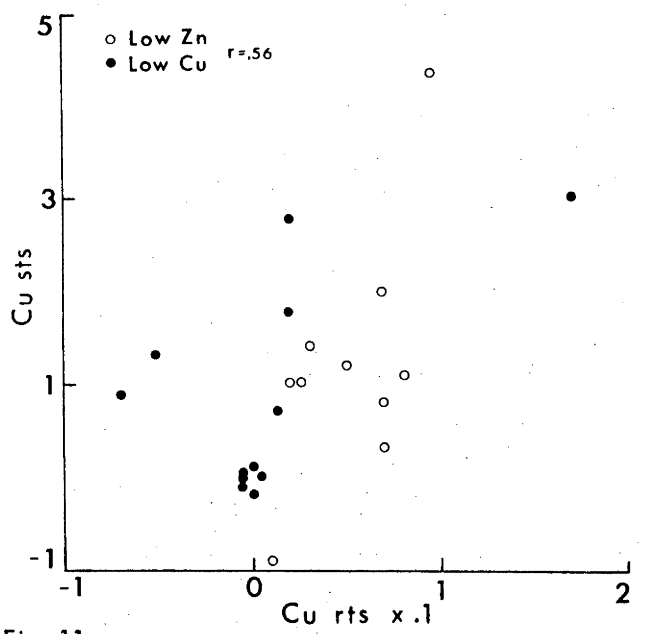


Fig 11

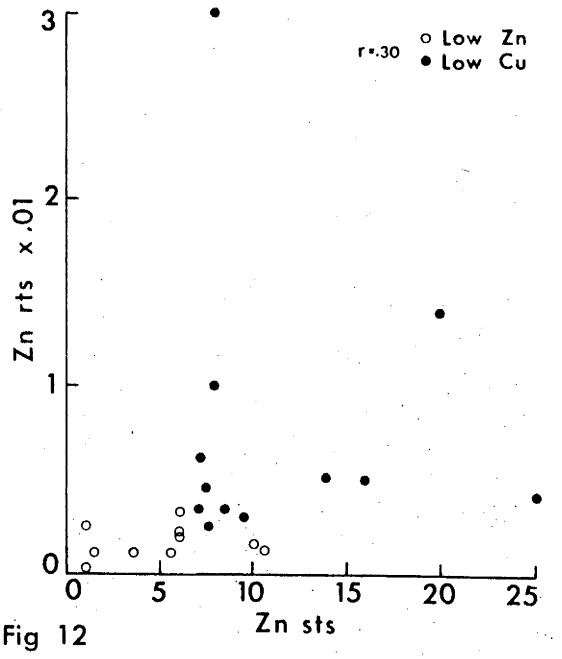


Fig 12

Secondly, it seems clear (Fig 7) that the samples shown were drawn from two populations. Seedlings pretreated with low or high Zn, which received deficient Zn levels throughout the measurement period, showed much lower rates of Zn movement to shoots than those which received low or high Cu pretreatments and adequate Zn throughout the measurement period (c.f. Table 2a). Similar effects applied with Cu. For Mn and Fe (Fig 8), which were in adequate supply throughout the experiment, there was no such separation. There did not appear to be any differences in the relationships due to shading, so data were not separated into those from shaded and unshaded seedlings.

The data were separated into two groups based on these differences and correlation coefficients between rates of metal movements to shoots estimated. The results are given in Table 4.

Table 4 Correlation coefficients relating rates of metal movement to shoots (μg metal per seedling per day per unit rate of change of fresh weight).

(a) Seedlings deficient in Cu

	Cu	Zn	Mn	Fe
Cu	1.00	.45	.45	.21
Zn		1.00	.71*	.90*
Mn			1.00	.93*
Fe				1.00

(a) Seedlings deficient in Zn

	Cu	Zn	Mn	Fe
Cu	1.00	.70*	.91*	.47*
Zn		1.00	.77*	.57*
Mn			1.00	.77*
Fe				1.00

* Significant with $p<.05$ at least

Table 5 Correlation matrix relating rates of metal movement to shoots and uptake by roots

		Movement to shoots				Uptake by roots			
		Cu	Zn	Mn	Fe	Cu	Zn	Mn	Fe
Shoots	Cu	1.00	.25	.45	.16	.56*	-.01	.57*	.07
	Zn		1.00	.82*	.66*	.17	.30	.67*	-.31
	Mn			1.00	.83*	.36	-.04	.55*	-.37
	Fe				1.00	.20	-.03	.28	-.39
Roots	Cu					1.00	.03	.54*	-.09
	Zn						1.00	.55*	.02
	Mn							1.00	.03
	Fe								1.00

* Significant with $p<.05$ at least

The results show that there were strong correlations between the rates of movement of Zn, Mn and Fe in both populations. When Cu was in deficient supply, its rate of movement to shoots did not correlate with rates of movement of other metals. When in adequate supply, the rate of movement of Cu significantly and highly correlated with the rates of movement of other metals.

It seems, therefore, that the movements of these four metals to shoots are closely related, if all are in adequate supply. When Cu was deficient, it was not able to kep pace with the other metals. As discussed earlier (Figs 5,6), there were indications that Zn was relatively more available in Zn deficient solutions than Cu was in Cu deficient solutions. This may explain why rate of Zn movement to shoots correlated with rates of movement of other metals, even when no Zn was added to the nutrient solution.

Figs 9 and 10 show the relationships between rates of uptake of metals by roots for selected metal pairs. Some negative rates of uptake by roots of Cu (Fig 9) and Fe (Fig 10) were observed. Figs 11 and 12 show the relationships between rates of movement to shoots and uptake by roots for Zn and Cu. There did not appear to be any separation into two populations with different results in the separate populations as there were in shoots, so these data were considered as a whole. The correlation matrix relating these variables for all metals is shown in Table 5 (p 127). The area enclosed by the dotted line in this Table was, of

course, constructed from the same data used to compile Table 4a and 4b, but pooled for both the Zn deficient and Cu deficient populations - this section of Table 5 is not of interest to this part of the discussion.

There was very little correlation between rates of uptake of Cu, Zn and Fe by roots, while Mn showed a relatively low ($r = 0.5-0.6$) correlation with Cu and Zn. There was also very little relationship between rate of movement of Cu, Zn and Fe to shoots with corresponding rates of uptake by roots. Again there were significant, although moderate, correlations between rates of Mn movement to shoots and rates of Cu and Zn uptake by roots.

Relationship between metal movement to shoots and transpiration

To allow for differences in growth rates with different treatments, transpiration rates over each measurement period were expressed as a fraction of the average fresh weight of seedlings over that period.

The effects of metal pretreatment and light intensity on transpiration rate per unit fresh weight are given in Table 6. Variances between samples were not homogeneous in either analysis, so appropriate techniques (Sokal and Rohlf 1969) were used to test the significance of differences between means.

The results suggested that pretreatment with high

Table 6 Main effects of seedling pretreatment and light intensity on transpiration rate (g water per seedling per day per unit average fresh weight over each measurement period). Results meaned over all harvests.

(a) Effect of pretreatments

Pretreatment				Significance (p<)
Zn 0ppm	Cu 0ppm	Zn 1ppm	Cu 1ppm	
2.09a	2.00a	4.01b	3.33ab	.001*

* Means which did not differ significantly have similar letters alongside them.

(b) Effect of light intensity

Light (%)		Significance (p<)
40	100	
2.73	2.63	NS

Table 7 Similar data to those of Table 6(a), but separated into data for the three time periods of the experiment.

Period (days)	Pretreatment			
	Zn 0ppm	Cu 0ppm	Zn 1ppm	Cu 1ppm
0 - 28	2.47	2.31	4.23	5.31
29 - 57	2.21	1.97	3.87	2.23
58 - 84	1.60	1.73	3.93	2.44

Zn or Cu increased transpiration rate. Table 7 gives the mean rates of transpiration for the different pretreatments over the three time periods. The significance of the interaction was not statistically tested because data making up this Table were unbalanced. It appears that for seedlings pretreated with high Cu, the higher transpiration rate was confined mainly to the first measurement period. The earlier results have shown that these seedlings were Cu deficient after that time. For seedlings pretreated with high Zn, the effect persisted over all time periods: earlier results showed these seedlings had sufficient, although declining, Zn levels throughout the experiment. Thus, it seems that transpiration rate was indeed reduced only when seedlings were Cu or Zn deficient.

Table 6b showed no significant effects of light intensity on transpiration rate. Thermohydrographs placed in full and 40% of full light showed no substantial effects of shading on temperature or relative humidity. Therefore, only reduced light intensity might have been expected to affect stomatal opening and hence transpiration rates. The lack of any effects is considered in later discussion.

To examine the relationship between rates at which metals moved to shoots and transpiration rate, data were separated into those for the two populations of seedlings receiving Cu deficient or Zn deficient solutions over the measurement period. Table 8 gives the correlation coefficients relating rates of metal movement to shoots and

transpiration rate.

The results showed no substantial relationship between rates of metal movement to shoots and transpiration rate.

Table 8 Correlation coefficients between rates of metal movement to shoots (ug metal per seedling per day per unit rate of change of fresh weight) and transpiration rate (g water per seedling per day per unit average fresh weight) for two populations of seedlings, one receiving Cu deficient solutions, the other Zn deficient solutions.

	Population	
	-Cu	-Zn
Cu	-.07	.18
Zn	-.36	.14
Mn	-.25	-.14
Fe	-.34	.08

DISCUSSION

Release of stored metals from roots to shoots

Excess Zn and Cu stored in roots during pretreatment with nutrient solutions containing high concentrations of metal, was released to shoots after seedlings were placed in Cu or Zn deficient solutions. This suggests that these metals are held in labile complexes within the root.

If, as discussed in previous papers, heavy metals are firmly bound within protein structure, then metal release would require at least unfolding of the protein chain, or

perhaps complete breakdown. Turnover of protein in meristematic tissue may be quite rapid. Helleburst and Bidwell (1964) found a turnover rate up to 0.5% of total protein per hour in expanding wheat leaves, while Steward and Bidwell (1966) found high turnover rates in rapidly growing carrot explant cultures. Racusen and Foote (1962) found that in senescing bean leaves turnover rates were also very large, suggesting that the physiological changes involved in senescence required rapid changes in the types of proteins being synthesized.

If rapid protein turnover can occur as a result of changing physiological conditions, it seems quite feasible to suggest that proteins may be synthesized specifically to store excess metal and may rapidly break down to release the metal under deficiency conditions. This also suggests that storage proteins may be synthesized as a specific defence mechanism against the excess and/or a safety mechanism against future deficiencies when excess metals are supplied to the seedling.

Mechanism of metal uptake by roots and release to shoots

The evidence from the correlation analyses relating the rates of uptake of Cu, Zn and Fe by roots gave no indication that the uptake of these metals was related when they were in adequate or deficient supply. These results disagree with the notion that uptake of all ions is controlled by the same mechanism when ions are in adequate

supply (see Introduction): for if they were, then in the long term steady state, their rates of uptake might be expected to correlate. There was evidence, however, that Mn uptake by roots may have been at least partially related to Cu and Zn uptake: Mn may share the uptake mechanisms of these two metals.

There were also no strong correlations relating rates at which metals were taken up by roots and the rates at which they were transported to shoots. This suggests that uptake by roots and release to the xylem are independent mechanisms and agrees with the present theory that there are two sites of active control of nutrients in the roots, one at the root surface and one at the stele.

There is little reason to assume that the theories advanced for ion uptake and release mechanisms based on studies with Cl^- and alkali metals should apply to the metals studied here. The long time period of this experiment also casts doubt on the validity of comparing this study to others which have been conducted only for a matter of hours. In the long term, substantial growth occurs and the whole physiological state of the seedling may change. This may change the seedling metal balance with time, although over any relatively short time period, close relations between the different nutrients may apply, in accord with the present theories of ion uptake. Changes in the seedling environment with time might also affect the metal balance of the seedling. In the solution cultures used here, nutrient concen-

trations were not constant, although there would have been adequate nutrient levels throughout the duration of the experiment unless seedlings were specifically supplied with metal deficient solutions. A complex flow system of solution culture, such as that developed by Asher et al (1965), would be necessary to ensure constant nutrient availability for more precise examination of these hypotheses.

A further problem with the technique used here, was that it was possible only to observe net rates of metal movement. While discussion has been limited to the one way root to shoot movement, reverse movement (hence negative rates of movement) was observed here. A number of workers have examined the mobility of elements in plants. K, Rb, Na, P, Cl^- and $SO_4^=$ generally are quite mobile (Lauchli 1972, Bukovac and Wittwer 1957) while Ca is phloem immobile (Marschner 1974). Bukovac and Wittwer (1957) found that Cu, Zn, Mn, Fe and Mo applied to leaves of bean were only partially mobile, but some of the applied metal did move out of the leaf. However, both Hill (1973) and Wood and Womersly (1946) have found that Cu may be held immobile in leaves of red clover and oats respectively. It seems likely that there was at least some movement of all the heavy metals assayed here in various directions in seedlings. This must throw further doubt on the interpretation of the purely net effects observed in this experiment.

There were strong correlations relating the rates at which Cu, Zn, Mn and Fe moved to shoots as long as the

elements were in adequate supply. This suggests that these metals have similar release mechanisms at the stelar barrier consistent with the concept of a non-specific mechanism controlling ion release from the cell when ions are plentiful (see earlier). When Cu was deficient, the specific control mechanism which operates at low nutrient concentrations may have controlled Cu release, hence there was no correlation with other metals. Unlike the uptake mechanism, the results suggested common release mechanisms for these metals to xylem when they were in adequate supply.

Relation of metal transport to shoots and the transpiration stream

There was no substantial correlation between transpiration rates and the rates of metal transport to shoots after removing the effects of growth from the data. The composition of the xylem sap may change seasonally and diurnally (Kramer 1969) so it is quite possible that the nature of the relation between metal composition of the xylem sap and the water content may change from time to time. In the short term then, over a matter of hours and under very constant environmental conditions, there might be a strong correlation between metal movement and transpiration rate as discussed earlier. But over the three month period of this experiment, this may not have applied.

On the other hand, while it has been agreed that mass flow carries the ions towards the leaves (Kramer 1969),

strong evidence has appeared that this is not the case for Ca in beans (Biddulph et al 1961, Bell and Biddulph 1963) and dogwood trees (Thomas 1967) or Sr in beans (Emmert 1965). For these elements, the xylem sems to behave as a chromatographic exchange column up which metals move by a series of exchange reactions. The nature of the exchange sites has not been elucidated (Bell and Biddulph 1963). Further work is necessary to relate these results to other elements. Certainly the heavy metals used here are chemically suited to behave in this fashion and Tiffin (1967) provides direct evidence that Mn, Co and Zn occur in xylem tomato sap as free cations. Hewitt and Gardner (1956) found that Zn was apparently adsorbed on the walls of vessels of grape stems by exchange with H^+ ions. There seems to be general concensus that the bulk of metals exist as free cations in the xylem stream (Biddulph 1959, Sutcliffe 1962). Fe is an exception: it has been found complexed with malate in soybean stem exudate (Tiffin and Brown 1962) and citrate in sunflower (Tiffin 1966). Movement of Fe might therefore be different to that of other metals because its binding and electrical properties are much altered by the chelation and because the availability of chelates will also affect its transport. If an exchange transport system operates, at least for Cu, Zn and Mn, in the xylem, ion movement might be quite independent of transpiration rate as was observed in this experiment.

Relation of light and metal deficiency to transpiration

Cu and Zn deficiency reduced transpiration from seedlings in this experiment. Apart from small amounts of water which may enter the xylem through gaps in the endodermal barrier (see Introduction), water must cross cell membranes at least twice in moving from the external solution to the xylem. Plant growth substances have been found to alter cell or membrane permeability to water (Tal and Imber 1971, Glinka and Reinhold 1971, Collins and Kerrigan 1973, Cram and Pitman 1972, Wood et al 1972). Co and Zn may reduce the volume of water exuding from tomato xylem (Tiffin and Brown 1962), while the hydrostatic pressure of water itself appears to induce changes in permeability of root cells to water (Kuiper and Kuiper 1974). Poor nutrition may affect stomatal opening (Price 1970) which may in turn affect transpiration rate.

Clearly there are physiological agencies, including plant nutrients, which affect transpiration rates and probably caused the effects observed here. These mechanisms are not as yet well understood.

Although reduced light intensity causes stomatal closure, there is debate as to how near to closure stomata must be before transpiration is reduced (Strafford 1965). It may be that the 40% of full light treatment here was not sufficient to close the stomata to the extent necessary to reduce transpiration rate. This would explain the lack of effect of reduced light on transpiration that was observed in

this experiment.

PAPER 5

HORMONAL CONTROL OF HEAVY METAL UPTAKE AND DISTRIBUTION IN SEEDLINGS OF *PINUS RADIATA* D.DON

ABSTRACT

IAA, GA, kinetin and ABA at 10^{-6}M were applied in nutrient solutions to two-week-old seedlings of *P. radiata*. The effects of the growth substances and aeration of the solution on uptake and transport of Cu, Zn, Mn and Fe for 12 weeks were studied. The uptake of Zn by roots was reduced by IAA and aeration. An integrated hypothesis for the control of Zn nutrition by auxin is proposed. Rates of Cu and Mn transport to shoots were increased by IAA, GA and ABA, suggesting distribution of these metals in the plant may be under hormonal control. Fe was not affected by growth substances or aeration. Uptake of all four metals by roots seemed to be controlled by related mechanisms as was release to the xylem stream.

INTRODUCTION

It has been common to think of (especially) meristematic plant tissues as "sinks" to which inorganic nutrients move in response to the need to maintain vigorous growth (Marschner 1974, Wareing 1970). Such "sinks" cannot

exist in isolation: some message must be transmitted through the plant to the sites which control nutrient uptake and transport so that nutrients may be available when and where they are required.

A growing body of evidence suggests that plant hormones influence the uptake and distribution of inorganic (and organic) nutrients in the plant and may act as the controlling factors in the nutritional balance of the plant. Thus, auxin applied to shoots of pea, poplar and bean caused phosphate to accumulate at the site of application. The effect of auxin was accentuated by adding cytokinin or gibberellin (Davies and Wareing 1965, Wareing 1970). Doerffling et al (1973) observed similar effects in sunflower and found that K also accumulated below the site of hormone application. They also found that abscisic acid reduced the effect of auxin. Wieneke et al (1971) found that foliar sprays of gibberellin reduced Ca absorption by shoots of pea and bean, while auxin increased Ca accumulation in shoots of bean but decreased it in pea. Neither hormone affected root uptake of Ca. Ashour et al (1974), on the other hand, were unable to find any effect of root applied gibberellin on Ca in roots or shoots of pea. Kannan and Mathew (1970) showed that foliar-applied kinetin and gibberellin affected the absorption and transport of foliar-applied Fe, Rb and phosphate in bean. The effects of the hormones differed in intensity and direction. Skoog et al (1938) applied auxin to the tips of decapitated corn and sunflower and found an increased rate of salt exudation from the xylem.

One specific hormonal effect that has been much studied is the control of stomatal opening by abscisic acid (ABA). ABA levels often increase markedly in leaves of droughted plants (Most 1971, Kriedeman and Loregs 1974, Beardsell and Cohen 1974). High levels of ABA reduce the K and sugar content of guard cells and the subsequent loss of turgor causes stomatal closure (Mansfield and Jones 1971, Penny and Bowling 1974). The mechanism of the hormone action is not known. ABA has increased, and kinetin decreased, the rate of K^+ and Cl^- transport in the xylem of maize (Collins and Kerrigan 1973,1974) but Cram and Pitman (1972) found opposite effects for ABA. Kinetin and ABA increased K, Ca and Cl^- flux into the roots of maize (Collins and Kerrigan 1974), but Cram and Pitman (1972) found no effect of ABA on root accumulation of K and Cl^-.

Little work has been done on the mechanism of action of plant hormones on nutrient uptake and transport. Wood et al (1971) showed that gibberellin increases the permeability of model phospholipid bilayers to glucose and chromate ions, suggesting that hormones may act by affecting membrane permeability. Rubenstein (1974) proposed that auxin increased Cl^- uptake by Avena coleoptiles by increasing extrusion of H^+ ions from cells and thus promoting ion uptake. These results agree with those of Marre et al (1974a,b) who showed both auxin and cytokinin increased H^+ extrusion from leaf and internode segments of squash, radish and pea.

Very little work has been done on hormonal control of heavy metals in plants. Skoog (1940) showed that Zn deficiency reduced the auxin level in tomato and other plants but that there was no effect of Cu or Mn deficiency on auxin levels. These results suggest an effect of the metal on the hormone rather than vice-versa.

Earlier studies in this series have examined the relation between heavy metals held in shoots and roots. But during this work, no information has become available about the factors that control the root-shoot metal balance. Given the possibility that control may be hormonal, the experiment reported here was designed to make preliminary investigations of the effects of exogenously applied plant growth substances on the uptake by roots and transport to shoots of Cu, Zn, Mn and Fe in P. radiata.

MATERIALS AND METHOD

Two-week-old P. radiata seedlings were transferred to nutrient solutions as described for earlier experiments. Nutrient solutions were the same as used previously but contained 0.1ppm Zn, 0.1ppm Cu and 0.01M $CaCl_2$. The $CaCl_2$ was found to inhibit pathogenic fungal growth which had caused damping-off of seedlings in earlier experiments (Heather pers. comm.). No attempts were made to estimate the effects of $CaCl_2$ on the nutritional status of seedlings.

Six treatments were applied at the time of

establishment: (i) control, (ii) aeration for 12 hours per day, (iii) 10^{-6}M indole-3-acetic acid (IAA), (iv) 10^{-6}M gibberellic acid (GA), (v) 10^{-6}M kinetin (6-furfuryl-amino-purine) and (vi) 10^{-6}M abscisic acid (ABA). Growth substances were supplied in the nutrient solutions and were changed at the fortnightly change of the solutions. Aeration was included as a treatment to determine if increased metabolic activity of the plant with increased oxygen availability would affect nutrition and/or seedling growth in a manner similar to the growth substances. The experiment was replicated twice.

Seedling were harvested at 0,4,8 and 12 weeks after establishment of the experiment. Roots were rinsed quickly in de-ionized water, seedlings were separated into shoots and roots, weighed fresh, dried at 85°C and re-weighed. The Cu, Zn, Mn and Fe contents of roots and shoots were detemined by atomic absorption spectrophotometry as described for earlier experiments. Shoot and root length (length of the longest root) were measured at each harvest.

RESULTS

Effects on seedling growth rates

Figs 1 and 2 show the effects of treatments on length and fresh weight of shoots and roots as a function of time. The results for oven dry weight gave identical trends to those for fresh weight, so only fresh weights are discussed (arbitrarily). Since all seedlings had the same

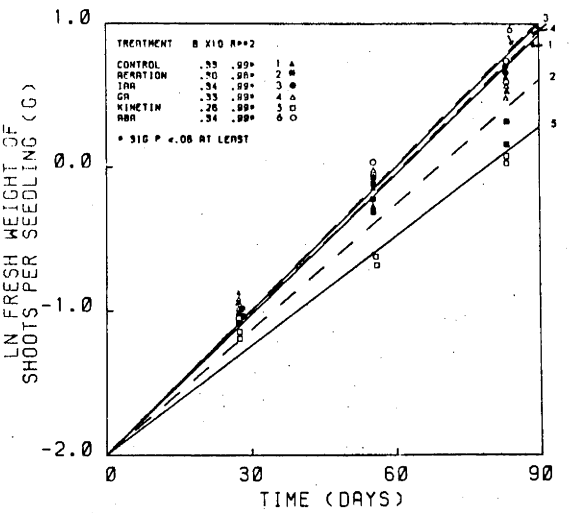


FIG 1A

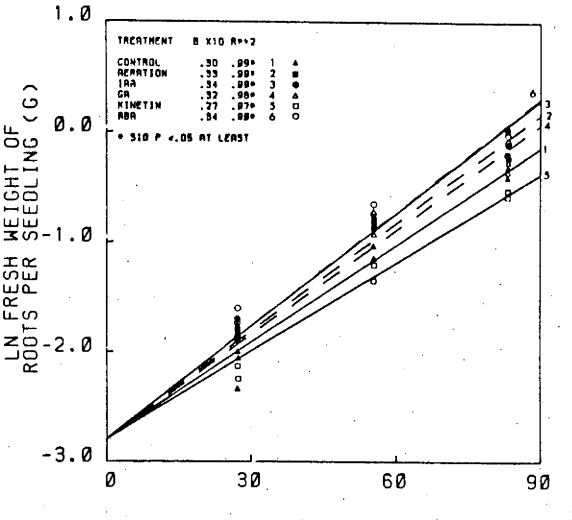


FIG 1B

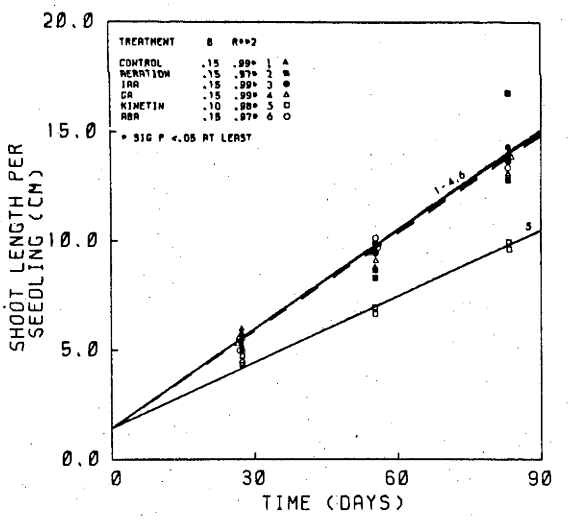


FIG 2A

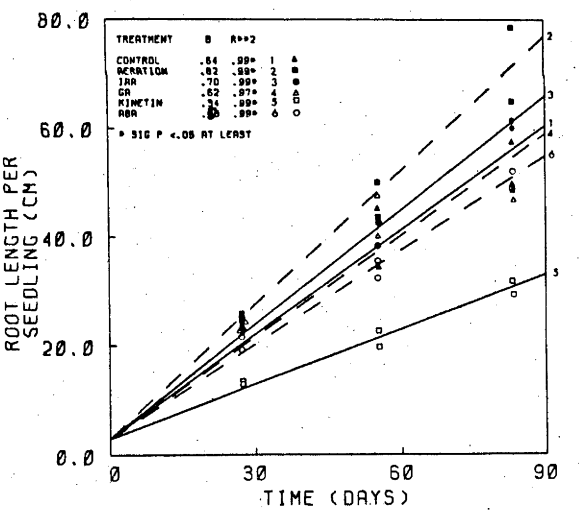


FIG 2B

Figs 1 and 2 Shoot and root fresh weight (Fig 1) and length (Fig 2) growth as a function of time for seedlings which received different treatments. B = regression coefficient, R**2 = proportion of variance explained by regression.

mean weight and length at establishment of the experiment, the regression equations were all forced through a common starting point. The slopes of the regression lines (b) and the proportions of the variances in the dependent variables explained by the regressions (R^2) are shown on the Figures. All regression equations were significant ($p<.05$ at least).

For shoot and root length (Fig 2) no conversion was necessary to convert the growth curves to straight lines. This has been observed for length growth elsewhere (Price 1970). Study of Fig 2 shows that very close straight line fits were obtained. For fresh weight (Fig 1), a logarithmic conversion was necessary to get a straight line fit. Attempts to fit other exponential growth relations (Ricklefs 1967) did not improve the fit. The fits obtained were not as close as might be desired, but fairly clearly they gave meaningful results in trying to determine the effects of treatments on growth rates. Values of R^2 tend to be inflated when regressions are fitted through a fixed intercept as in this case (Spurr 1952). Hence the values shown in Figs 1 and 2 probably tend to exaggerate the closeness of the straight line fit.

Covariance tests of the significance of the differences between slopes of the regression lines in each of the four sections of Figs 1 and 2 showed there were highly significant differences ($p<.001$) in all cases. For root length (Fig 2b) seedlings grown in kinetin showed a significantly lower growth rate (i.e. the regression line for

kinetin had a lower slope) than those for other seedlings (differences tested with the simultaneous test procedure with p=.05 - Sokal and Rohlf 1969). Roots of kinetin treated seedlings were much thicker than those in other treatments as well as shorter. This suggests kinetin induced much cell division in roots at the expense of cell elongation. Aeration significantly increased the rate of root length growth when compared to other treatments. Other treatments had no significant effects.

Rate of shoot elongation was significantly lower in kinetin treated seedlings than in seedlings with other treatments (Fig 2a). The other treatments did not differ significantly from one another. No tests were made to determine if this kinetin effect was due to increased cell division as a result of kinetin transport from root to shoot or was a result of the natural tendency for root and shoot growth to correlate because of their interdependence (Price 1970).

Kinetin significantly reduced the rate of root and shoot fresh weight growth (Fig 1). But because these seedlings had thicker and/or shorter shoots and roots than those with other treatments, the effect on weight was not as marked as that on length. The other treatments had no significant effects on rate of root or shoot fresh weight growth.

Effects on metal uptake by roots and transfer to shoots

Figs 3-10 show the relation between the rates at which metals moved to shoots or were taken up by roots and the rate at which fresh weight changed over each time period. Data for all three measurement periods are shown in each Figure. The rate of change of fresh weight was used as the independent variable as it seemed reasonable to assume that metals would move to shoots or be taken up by roots in response to new growth over each period. The regression coefficient (b), intercept (a), the proportion of the variance in the rate of metal movement explained by each regression (R^2) and the significance of each regression is also shown on the Figures. With a few exceptions, the regression equations were highly significant and explained 90% or more of the variation in the dependent variables.

The effects of the treatments on rates of metal movement to shoots and uptake by roots can be judged by the slopes of these relationships. The significance of the differences between slopes can be tested by normal covariance analysis. However, as discussed in the previous experiment, and later in this paper, the rates at which the various metals move to shoots or are taken up by roots may, at least partially correlate with each other because similar factors may, at least in part, control their uptake or transport in the seedling. This means that an apparent effect of the treatments on one metal may reflect only their effect on another metal, because the rate of movement of the first

Figs 3-10 Rates of movement of metals to shoots (Figs 3-6) or uptake by roots (Figs 7-10) as a function of rate of change of fresh weight for seedlings which received different treatments. A = regression constant, B = regression coefficient, R**2 = proportion of variance explained by the regression.

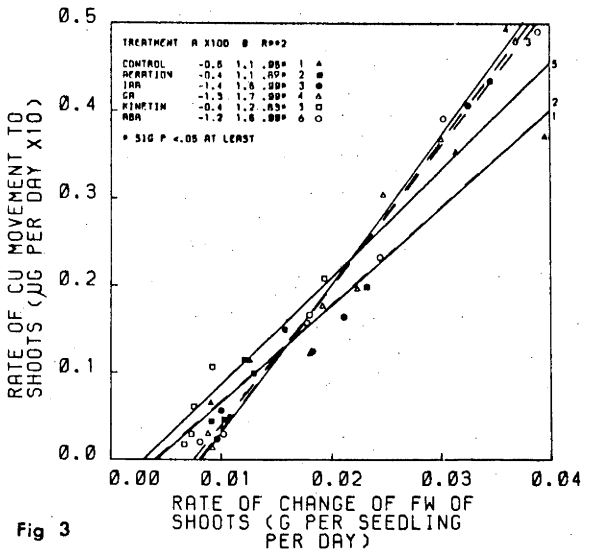


Fig 3

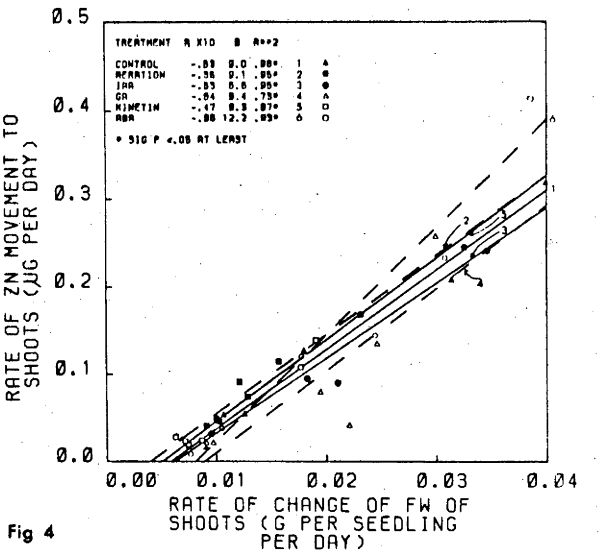


Fig 4

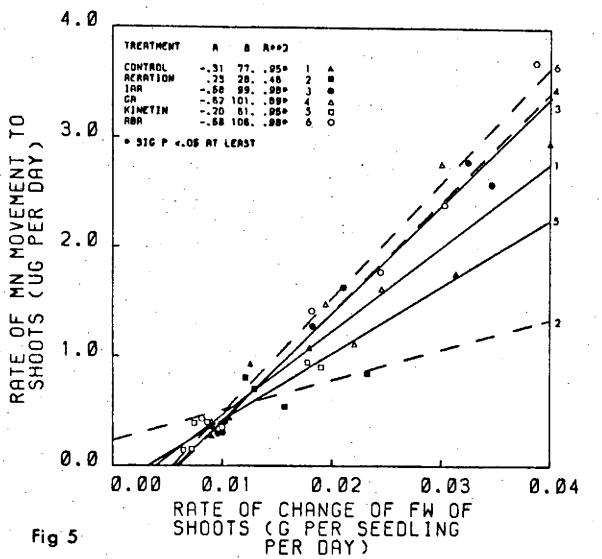


Fig 5

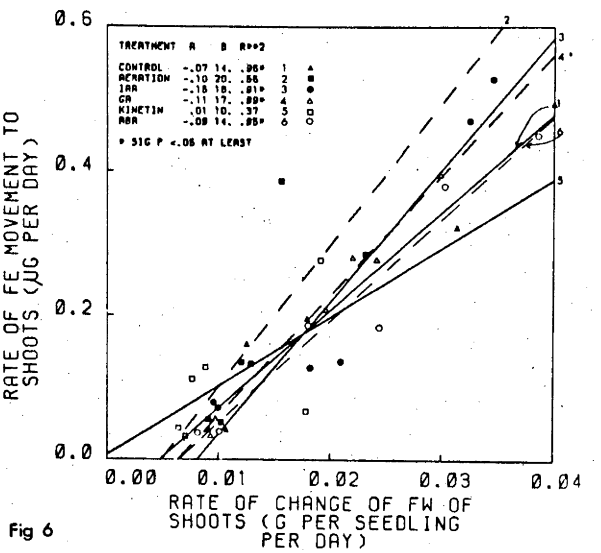


Fig 6

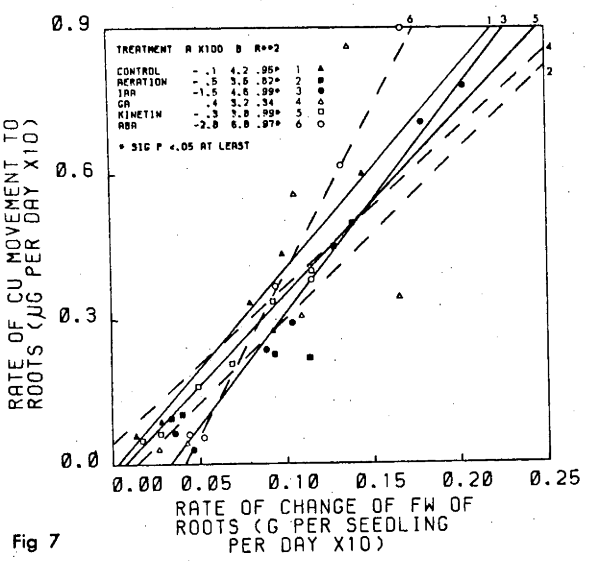


Fig 7

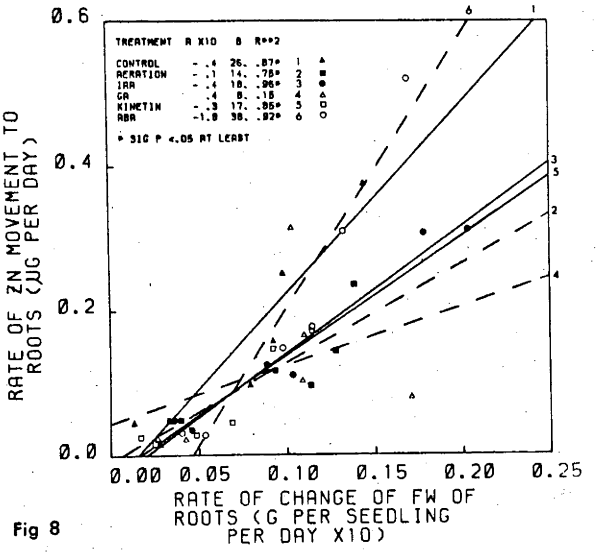


Fig 8

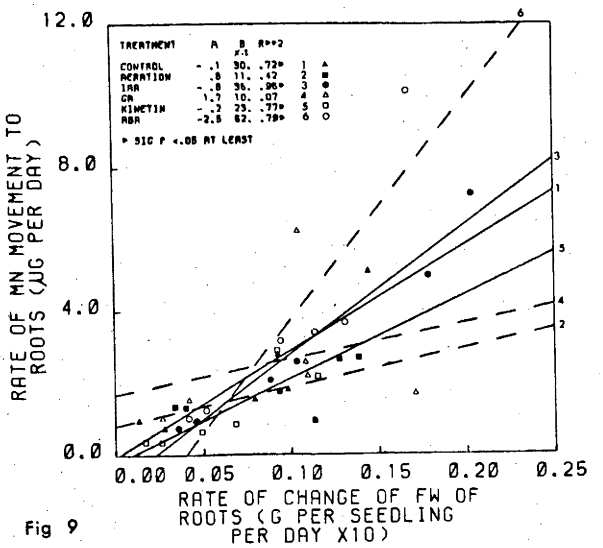


Fig 9

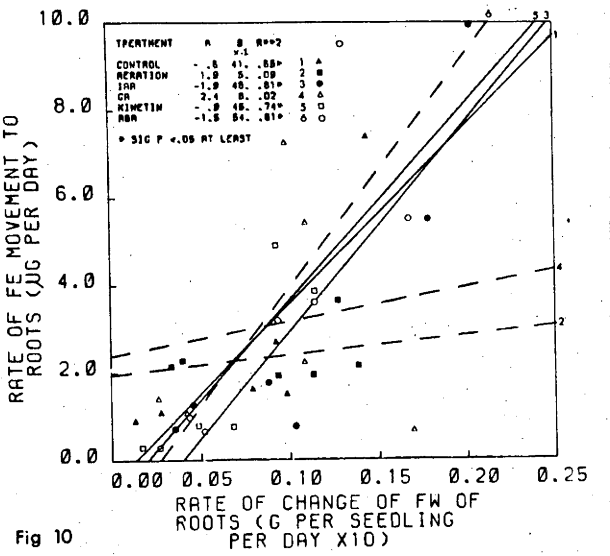


Fig 10

metal was at least partially correlated with the rate of movement of the second. Normal univariate statistics treat each metal individually and take no account of these correlations (covariances) between the various parameters measured. Multivariate statistics solve these difficulties by taking as much account of the $p(p-1)/2$ covariances among the p variables measured as of the p variances which are individually examined by univariate techniques. Cooley and Lohnes (1971) and Hope (1960) provide useful introductions to these techniques.

Multivariate analysis of covariance works in much the same way as univariate covariance except that a p x p matrix of sums of squares and cross products between the p variables measured replaces the p individual sums of squares found in univariate analysis. One matrix is estimated for each source of variation (among, within and total) and the determinants of these matrices are used as multivariate measures of variance for significance tests in a very similar way to the use of variance in univariate tests. With multivariate techniques, the calculations are generally so arduous, that use of a computer is essential. Cooley and Lohnes (1971) provide a useful set of programmes.

The four variates measured in this experiment were the rates of movement of the four metals to shoots or the rates of uptake of the four metals by roots. Separate analyses were done for root and shoot data. The multivariate test of the significance of the differences between slopes of

regression lines was significant for shoots ($p<.01$, data from Figs 3-6) and roots ($p<.05$, data from Figs 7-10). That is to say, there was evidence that there were effects of at least some of the treatments on rates of uptake or transport of at least some of the metals.

As with univariate analyses, a second step must now be taken (the 'a posteriori' test) to determine which treatments actually had effects. With the multivariate analyses, another step must be taken to determine which of the measured parameters were affected by which of the treatments. Discriminant analysis performs these tests by finding several (in fact p, or if g (the number of treatments) is less than or equal to p, then g-1) 'discriminant functions', each independent (orthogonal) of each other. From these functions can be deduced, as will be described below, which treatments affected which of the parameters measured.

In both the analyses here, only one discriminant function was significant ($p<.05$ at least). Table 1 shows the correlation coefficients relating the data (transformed by the discriminant analysis) for each metal to the discriminant functions. If the correlation coefficient for any parameter is high, it suggests that there were effects of treatments on that parameter. The percent trace shown in the table shows the proportion of the discriminating information available in the data that was extracted by that discriminant function. Clearly, both of the functions extracted here contained the

bulk of the discriminating information available.

The results of Table 1 suggest that in shoots, treatments affected the rate of movement of Cu and Mn, both of which correlated moderately to highly with the discriminant function. It appears there were no significant effects of treatments on rate of movement of Zn or Fe to shoots. In roots, the major effects of treatments appears to have been only on rate of uptake of Zn by roots, and rate of uptake of Cu, Mn or Fe was little affected.

Table 1 Correlation coefficients (factor pattern) between parameters measured and the significant discriminant functions determined in the two analyses performed here. Rates of metal movement measured as μg metal per seedling per day, independent of effects of rate of fresh weight change.

		Shoots	Roots
Percent trace		79.	61.
Metal	Cu	.72	.44
	Zn	.13	.82
	Mn	.76	.60
	Fe	.15	.40

Figs 11 and 12 show the slopes of the regression lines (adjusted by the weightings given them by the first discriminant function), relative to the control, for seedlings receiving different treatments. As discussed above, Fig 11 represents the effects of treatments on the rates of Cu and Mn movement to shoots, while Fig 12 represents the effects on rates of Zn uptake by roots. A 95% confidence

interval was estimated to indicate which treatments had significant effects. This interval was formed by pooling the variances of the results for each individual treatment (variances were found using the programme of Cooley and Lohnes (1962) and were found to be sufficiently homogeneous to allow pooling) to form the equivalent of a least significant difference in univariate analysis of variance. Thus, all the data of Figs 3-10 have been reduced to those of Figs 11 and 12 by the multivariate analysis.

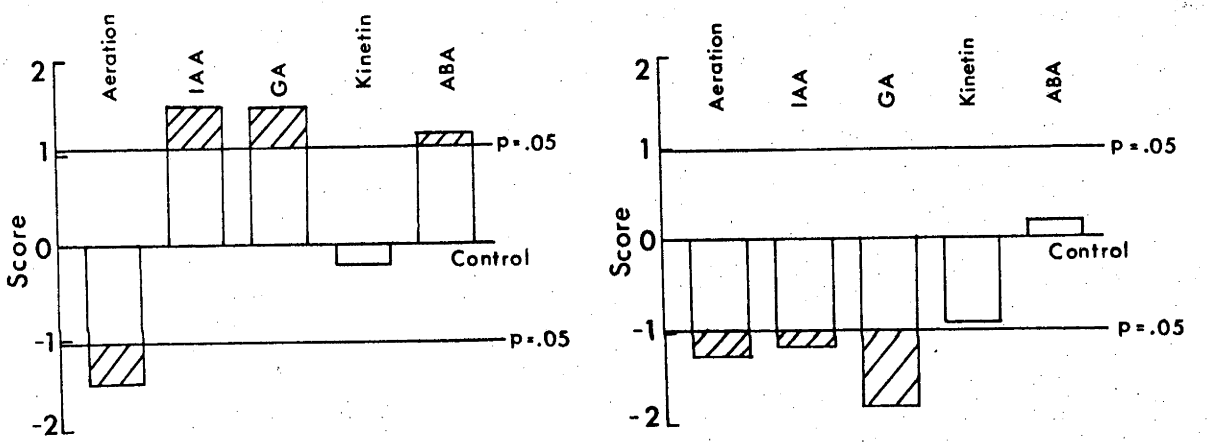


Fig 11 (left) and Fig 12 (Right) Effects of treatments relative to control on rates of Cu and Mn movement to shoots (Fig 11) and rate of Zn uptake by roots (Fig 12) as determined by discriminant analysis. The scores shown are arbitrary units determined by the conversions of the analyses and are for the first discriminant function only in each analysis. The confidence limit (p=.05) about the control is shown.

The results of Fig 11 suggest that the rate of Cu and Mn movement to shoots was reduced in aerated seedlings. However, Fig 5 shows that there was no significant relation between rate of Mn movement to shoots and rate of change of fresh weight - the reason for this was not apparent. Fig 3

shows that the slopes of the lines relating rate of Cu movement to shoots for control and aerated seedlings were virtually identical. The apparent effect of aeration on Cu and Mn movement was, then, probably an artefact due principally to the non-significance of the regression for Mn. Fig 11 does indicate, however, that IAA, GA and ABA all significantly increased the rate of movement of Cu and Mn to shoots and kinetin had no effect.

In roots (Fig 12) the only substantial effect was on rate of Zn uptake where aeration, IAA and GA appeared to reduce uptake. Fig 8 shows that the regression relating rate of Zn uptake and rate of change of root fresh weight for seedlings treated with GA was not significant - the reason for this was not clear. This suggests that only aeration and IAA had real effects on Zn uptake.

Relation between rates of uptake and transport of metals

As discussed above, certain treatments had effects on rates of uptake by roots and movement to shoots of certain metals. At the same time, the rates of uptake or movement of any of these metals may correlate, at least partially, with rates of uptake or movement of the other metals if their uptake mechanisms are related.

The variation in the data, as a result of treatment effects, provided an opportunity to test whether the rates of uptake and movement of the different metals were related. To

remove effects of growth, rates of metal uptake by roots or transport to shoots were expressed per unit rate of change of fresh weight of roots or shoots respectively. The correlation matrix relating rates of uptake of the various metals by roots and rate of transport to shoots for data from all treatments and over all time periods is shown in Table 2.

Table 2 Correlation coefficients between rates of metal uptake by roots and transport to shoots (µg metal per seedling per day) per unit rate of change of fresh weight (g per seedling per day).

		Movement to shoots				Uptake by roots			
		Cu	Zn	Mn	Fe	Cu	Zn	Mn	Fe
Shoots	Cu	1.00	.61*	.64*	.69*	.73*	.39	-.02	.11
	Zn		1.00	.61*	.44*	.66*	.76*	.37	.33
	Mn			1.00	.31	.58*	.47*	.32	.20
	Fe				1.00	.40	.10	-.22	-.11
Roots	Cu					1.00	.77*	.47*	.49*
	Zn						1.00	.71*	.56*
	Mn							1.00	.70*
	Fe								1.00

* Significant with $p<.01$ at least

There were significant, but only moderate, correlations between rates of uptake of all four metals by roots and also between rates of transport to shoots (except for rates of Fe and Mn movement to shoots). Rates of Cu and Zn uptake by roots correlated significantly with rates of transport of one or more metals to shoots, but in general, rates of uptake of metals by roots were little correlated with

rates of transport to shoots.

DISCUSSION

Growth effects

Kinetin was the only hormone that significantly affected growth rates of seedlings during the experiment. This was probably due to increased cell division at the expense of cell enlargement which produced shorter and thicker roots and shoots. This is the general growth response of plants to cytokinins (Huber and Sankla 1974, Price 1970). The other hormones had no significant effects on growth rates. Growth responses to GA and IAA are known to vary widely depending on a number of factors, e.g. concentration of hormone, age of tissue, type of tissue, temperature, light availability; sometimes growth promotion occurs and sometimes inhibition (Price 1970, Paleg 1965, Strafford 1965). ABA generally inhibits growth of stems and roots (Addicott and Lyon 1969) but no such effect was observed here. The concentrations of growth substances used here were chosen relatively arbitrarily but other work has shown these levels to be generally active in affecting growth (Jenkins 1971, Price 1970). No tests were conducted to determine if the hormones retained their activity throughout each fortnightly period between the change of nutrient solutions nor to determine if they were actually taken up by the seedlings. Since there were consistent effects of the growth substances on specific plant nutrients, it seems likely they

were active for at least the bulk of each period and must have been absorbed by roots. The lack of effects of IAA, GA and ABA on growth rates may then be due to chance combinations of plant and environmental conditions under which these substances were not active in affecting growth rates. Auxin, gibberellin, cytokinin and abscisic acid activities have been identified in P. radiata (Jenkins 1971, Shepherd 1965) but little work seems to have been done to determine the specific effects of these growth substances on growth of this species.

Aeration increased the rate of elongation of roots but had no effect on elongation of shoots or weight of shoots or roots. This result agrees with those of Zinkan et al (1974) who studied the effects of oxygenation of the nutrient solution on growth of seedlings of jack pine, black spruce and white spruce. They found that less than 2ppm O_2 in solution reduced weight and length of roots and shoots, but with 3.3ppm O_2, only length of roots was reduced. This suggests that root elongation is more sensitive to O_2 level than shoot elongation or root or shoot weight. The O_2 levels in non-aerated solutions in this experiment must have been adequate for maximum shoot extension and shoot and root weight growth but less than adequate for maximum root extension. The reasons for differential length and weight growth response are not known.

Effects of growth substances on metal uptake and transport

Rate of uptake of Zn by roots was reduced by application of IAA in the nutrient solution. Root uptake of the other metals was not affected. This result expands on the outstanding work of Skoog (1940). He found that in the early stages of Zn deficiency in tomato, sunflower, corn, alfalfa and apricot, before growth declined, there was a marked reduction in auxin level (as measured by the Avena curvature test) in all parts of the plant. He showed that the Zn deficient tissue had stronger oxidising properties than normal tissue and inactivated applied IAA. As well the relief of Zn deficiency stimulated the synthesis of auxin. Skoog was unable to explain his results biochemically. Subsequently it has been shown (Hewitt 1963, Nason et al 1952) that not only is Zn a co-factor to tryptophan synthetase (tryptophan is a precursor in IAA synthesis (Hughes and Genest 1973, Brady 1973)) but also that Zn deficiency increases the activity of auxin oxidase or peroxidase which degrade IAA (Harborne 1973). These observations explain the effects of Zn deficient tissue on IAA levels that Skoog recognised, but was unable to explain.

Skoog's work and the result reported in this experiment that applied IAA reduced Zn uptake by roots suggest there is an integrated control mechanism for Zn nutrition. The plant responds to Zn deficiency by reduced IAA synthesis and increased IAA degradation. The decreased IAA level in the plant then has two effects. First, IAA

inhibition of Zn uptake by roots is decreased so the root has the maximum chance of taking up any Zn available in the external medium. Secondly, IAA induced growth is reduced so that the plant does not grow vigorously when an essential nutrient is unavailable.

Skoog showed that Cu and Mn deficiency did not affect auxin levels in the plant until well after growth had declined. This suggested that the effects of these metals on auxin was secondary and did not occur until after the initial lesions due to metal deficiency had reduced growth and the overall metabolism of the plant had been severely disrupted. No effects of applied IAA (or other hormones) on uptake of Cu, Mn (and Fe) were observed in this experiment, suggesting that neither auxin (nor other hormones) control their uptakes by roots, at least under the conditions of this experiment.

Further work is necessary to elucidate the mechanism by which IAA inhibits Zn uptake by roots and to verify that all the effects on Zn and auxin implied by this hypothesis for Zn control can be observed in the one species.

Rate of Zn uptake by roots was also reduced by aeration of the nutrient solution. Skoog (1940), in passing, mentions that Zn deficiency symptoms in sunflower became extreme only in aerated solutions. Zinkan et al (1974) showed that foliar N and K levels were reduced by low O_2 in the nutrient solution while Mg, Ca and Fe levels increased. It is not clear how these effects occur.

Rates of Cu and Mn transport to shoots were increased by additions of IAA, GA and ABA to the nutrient solution. This suggests there may be hormonal control of the distribution of these elements in the plant. These three substances are known to interact very strongly with each other in control of growth processes (Addicott and Lyon 1969, Paleg 1965, Addicott 1972) and if all three were involved in control of Cu and Mn transport in plants, the interaction and balance between them may be very complex. Further work is necessary to expand on these observations. In particular, the relation between these metals and endogenous levels of these hormones requires study. The relatively arbitrary choice of concentrations of applied hormones in this experiment may have led to non-physiological concentrations in the seedlings and hence abnormal effects.

No effects of hormones on Fe uptake by roots or transport to shoots were observed in this experiment. Fe nutrition may be controlled by other means in plants or the conditions of the experiment may have been such that no effects on Fe appeared.

Relation between rates of uptake and transport of the four metals

The relation between the rates of uptake of the four metals by roots and transport to shoots was studied to compare with the results found in the previous experiment. The essential difference found in this experiment was that

the rates of uptake of all four metals by roots correlated significantly, although the correlations were low to moderate (0.4-0.7). This suggests that the uptake mechanisms for these metals are at least related to each other. This agrees with the curent concept of common nutrient uptake mechanisms as discussed in the previous paper.

The rates of transport of all four metals to shoots correlated with each other (with the exception of Fe and Mn) although much less strongly than in the previous experiment. This implies that the release mechanisms for these metals to the xylem stream are allied. There were significant correlations relating rates of metal uptake by roots and transport to shoots, particularly between Cu, Zn and Mn. Some similar correlations were observed in the previous experiment, mainly with Mn. If uptake of metals by the roots and release to the xylem stream are physically separate machanisms (see discussion in previous paper), then the rates of uptake and release might be expected not to correlate, although it is possible that under steady state conditions they may, fortuitously, do so. Further work is necessary to clarify the meaning of these rather variable effects.

Because of the correlations which exist between rates of uptake and transport of metals, any hormonal control mechanism of one, such as discussed above, may apparently affect another because their uptake or release mechanisms are similar or related. The value of multivariate techniques of analysis to allow for these relationships and identify actual

effects from pseudo-effects is obvious.

CONCLUSION

THE CONTROL OF HEAVY METAL NUTRITION OF PLANTS - AN INTEGRATION OF THIS WORK

INTRODUCTION

In preparing the five preceding papers, it was attempted to make each complete in itself, with a rounded discussion, but with minimum reliance of one paper on the others. As such, all the results of the experimental work here have been discussed in detail at some stage of this work. The aim of these conclusions is not to reproduce in summary form all the details of the results, but rather to present an integrated description of the current concepts of plant nutrition by following the path of inorganic nutrients from entry at the root till utilization in the shoots. Where the results of this study have impinged on this pathway, their relation to the overall plant nutrition process is examined.

Work in plant nutrition, as with most plant studies, varies greatly in the species and conditions used by different authors. Neither heavy metals nor conifers feature to any great extent in such studies. So this review is limited by the generalizations drawn from work with widely divergent species and study conditions which have been

applied to the current situation.

This concluding paper first discusses the general problems of experimental techniques encountered in this work. Then follows an examination of the path of ion transport in plants, a discussion of the metabolic role of heavy metals and finally a discussion of the possible role of plant hormones in integrating control mechanisms for nutrient uptake and dispersion. Some small scale experimental work, not previously reported, is also discussed. Where appropriate, avenues for further research are suggested.

NOTES ON THE EXPERIMENTAL TECHNIQUES

Solution cultures

When approaching plant nutrition purely from the point of view of plant responses, it seems essential to use solution cultures. Under such conditions there is no question of the availability of nutrients to the plant, provided of course the solution is not abnormal in e.g. pH or nutrient constituents. On the other hand, in soil culture the soil itself may markedly alter nutrient availability (see below) and hence plant responses. The entire root system of the plant is easily recovered from solution culture, whereas it is impossible to extract a root system from soil without damage as soil particles are removed. However, in longer term experiments, the management of solution cultures is time consuming since solutions require changing at least fortnightly and often much more frequently. The ultimate in

complexity are the continuous flow systems such as those of Asher et al (1965). If plants are to be grown to a large size, the logistics of solution cultures become even more difficult (cf. Hewitt 1966)

When dealing with micronutrients, it is very difficult to find support media sufficiently free of the nutrient involved to produce nutrient deficiencies. Barker (1973) was unable to produce Zn deficiencies in artificial media (pearlite or vermiculite) or in acid-washed sand because of Zn contamination. Solution cultures are much easier to manage from this point of view. Both Zn and Cu deficiencies of P. radiata were obtained in the solution cultures used in these experiments (Frontpiece and Paper 1), but the solutions were certainly not free of Zn or Cu since quite substantial uptake of both elements occured from deficient solutions (Paper 4). It seems essential to use purification procedures to rid commercial macronutrient salts of heavy metal contaminants as shown by Barker (1973) who had difficulty in obtaining consistent Zn deficiency symptoms in P. radiata with unpurified salts. Co-precipitation of heavy metal contaminants with $Mg(OH)_2$ (Munns and Johnson 1960) was consistently successful in this work. Other purification techniques have been developed (Munns and Johnson 1960, Price and Vallee 1962); these are often time consuming but may be essential if even purer solutions are to be obtained than those used here.

Contaminating microorganisms

Algal growth is a common problem with solution cultures. Initially in this work, it was thought that weekly changes of nutrient solutions would be sufficient to prevent this, but it proved not to be so (Paper 1). Painting the culture pots black to exclude light was completely successful in combating this problem.

Fungal pathogens are problems with any plant study and caused considerable trouble in this work. Other work in this laboratory (Prajapati pers. comm.) has shown Pinus sp. seedlings in solution culture are susceptible to active concentrations of common fungicides. As well, for micronutrient studies, these substances may be contaminated with metal salts leading to further purification problems. To control the pathogens encountered, 0.01M $CaCl_2$ added to the nutrient solutions proved very effective (Paper 5), but such high concentrations of Ca^{2+} or Cl^- ions may affect the nutritional status of seedlings. Where pathogenic fungi affected seedling growth, allowances were made for these effects in interpreting results.

Another common problem with nutritional work in plants is the presence of mycorrhizal fungi. Mycorrhizal roots often show much greater accumulation of inorganic nutrients than non-mycorrhizal roots (Harley 1969). Much work on mycorrhizal uptake has been done with soil cultures rather than solution cultures. A number of proposals for the mechanism by which mycorrhizae affect nutrient uptake involve

increased soil availability of ions, although other mechanisms, involving increased efficiency of uptake mechanisms of mycorrhizal roots have been proposed (Harley 1952). Increased phosphorus uptake by mycorrhizal roots has been much studied (Harley 1969) and has been observed in P. radiata seedlings (Stone 1949). Uptake of alkali cations and Fe has also increased in mycorrhizal roots (Routien and Dawson 1943, Melin et al 1958). In the experiments reported here, there was no evidence of any mycorrhizal associations with roots in microscopic sections of roots. Nor were any branched roots, typical of mycorrhizae, observed. It seems likely, therefore, that metal uptake studied in these experiments was not mediated by any mycorrhizal association.

Aeration of nutrient solutions

Hewitt (1966) has summarised extensive information on the need for aeration of solution cultures. In the experiments reported here, there were no immediately apparent deleterious effects of lack of aeration on P. radiata seedlings. In the one experiment (Paper 5) where aeration was applied as a treatment, root growth was increased. This agreed with other results for pine (Zinkan et al 1974) which suggested that root growth was more sensitive to oxygen level in the nutrient solution than shoot growth, probably because shoots obtain oxygen from the air. It was clear, however, that the effects of aeration on seedling growth in the experiment here was only minor. In general, it seemed there

was sufficient oxygen in nutrient solutions over each fortnightly period to ensure seedling health was satisfactory.

In experiments with clover (Pirie 1973), there was an interaction between Cu level in the nutrient solution and aeration in determining the amount of soluble amino-N in seedlings. In non-aerated solutions, increasing Cu from 0 to 0.7ppm decreased amino-N in the plant, whereas in aerated solutions amino-N increased with more than 0.07ppm Cu in solution. Effects on individual amino acids, argininie, histidine and β-alanine and on ethanolamine were also discussed. Changes of this nature in the chemical constitution of the plant suggest that there may well be effects of aeration on plant nutrition which should be accounted for in nutritional work. Substantial research would be needed to fully examine these effects.

Time-scale of experiments

As was discussed in Paper 4, interpretation of the results in terms of the theories of mechanisms of nutrient uptake by cells was difficult in long term experiments such as these. The nature of some of the experiments - the relation between nutrient levels and growth (Paper 1), the nutrient content of protein in seedlings (Paper 2) and the release of stored metal from roots (Paper 4) - required that they be long term; the problems of interpretation in Paper 4 arose in discussion of results that were secondary to the main thrust of the paper. In determining the fine details of

the mechanisms of interaction between nutrients in uptake (Papers 1,2), the effects of growth substances (Paper 5) and the workings of the uptake mechanism (Papers 4,5), short term experiments (hours) may be essential as the kinetics of these processes may change over a matter of hours (Laties 1969). However, effects in the short term may not persist in the long term and have overall major effects on the general nutritional state of the plant. Given the purposes of this work (Introduction p 3) long term studies of these effects have substantial relevance.

HEAVY METAL NUTRITION

Nutrient availability in soil

The availability of inorganic nutrients at the root surface determines the ability of the plant to absorb nutrients, irrespective of any control mechanism of the plant itself. The determination of the availability of nutrients to plant roots was not the subject of this work. But a brief summary of the factors which affect nutrient availability when the plant is growing in its native environment, the soil, is presented here for completeness, because these are the first control mechanisms which affect plant nutrition. Hodgson (1963) summarised the principal factors affecting, particularly, micronutrient availability in the soil and his review forms the basis of discussion here.

All the micronutrient elements may be bound within soil minerals during their formation in the geologic past.

As these minerals weather, forming the soil itself, the bound elements may be slowly released to the soil solution: the speed of this process determines the ultimate availability of inorganic nutrients to plants. However, metals released in this way may subsequently become completely unavailable by occlusion inside secondary minerals formed during weathering processes (ironstone gravels, for example, are not uncommon in leached Australian soils). As well, metals may be adsorbed more or less firmly at the surface of soil particles. Thus, Fe and Mn may, under the right conditions of aeration, precipitate as their highly insoluble oxides or phosphates onto the surface of soil particles. The subsequent release of these metals from these compounds depends on the pH, oxidation potential, temperature and availability of soluble complexing agents in the soil solution. Electrostatic forces too may firmly attach metals to the surface of soil particles after exchange reactions: the conditions of the soil solution will affect the subsequent availability of these elements also. Organic matter, which often has a high cation exchange capacity, may strongly bind metals: Cu, in particular, may be very firmly held in organic form.

The physical location of nutrients in the soil profile may also affect their availability to the plant. Elements may be distributed in the soil profile in a characteristic manner depending on their chemistry and the chemistry of the soil as leaching waters move them down through the profile. The rooting characteristics of a

particular plant will then determine if it can reach the nutrient pool in the soil. Conversely, the physical characteristics of the soil may prevent plant roots penetrating and reaching available nutrient pools.

Microorganisms, both bacterial and fungal, have marked effects on nutrient availability in soil. They may release nutrients stored in organic material by decomposing the organic matter or they may release metals from oxidised forms by reduction. They may reduce nutrient availability by incorporating nutrients for their own use or by oxidising metals to less available forms: Mn and Fe in particular have their oxidation states controlled by microorganisms. Microorganisms may indirectly affect nutrient availability by affecting the pH or oxidation potential of the soil solution.

Plants themselves may modify the soil environment. Firstly, they may compete with other plants for available nutrients and hence alter the availability of nutrients to their competitors. Secondly, many plants exude a great variety of compounds in large enough quantities to affect nutrient availability. Exudates may affect the activity of microorganisms, which may in turn affect nutrient availability, or they may chemically affect metal availability.

A nutrient solution is, of course, the ideal environment for the plant. Nutrients are available in a suitable form for absorption and the root does not have to seek out water or nutrients as it does when the plant is growing in soil. The remaining sections of this paper

discuss the control of plant nutrition, given that the inorganic ions have already reached the root surface and are available for uptake. . .

Nutrient uptake at the root surface

Initial uptake of inorganic nutrients at the root surface occurs mainly via root hairs (Kramer 1969) and is under both active and passive influences. There is free (passive) access for water and nutrients into the free space of root tissues. Free space has two parts, the water free space, which consists of inter-cellular spaces and parts of the cell wall (Price 1970) and Donnan free space which is defined as that part of the tissue available to the external solution but which is under the influence of non-mobile anions (Jennings 1963). There has been very active and continuing debate as to the exact location of Donnan free space and, in particular, whether the plasma-lemma is freely permeable to inorganic ions. Older work (Briggs and Robertson 1957) considered the tonoplast to be the major barrier to active ion entry and therefore the cytoplasm itself may have constituted the Donnan free space. In light of the more recent hypotheses of dual active uptake mechanisms (p 107), it has been proposed by workers who suggest that the second, non-specific, mechanism operates at the tonoplast (Latles 1969) that the plasmalemma is freely permeable to ions at high external salt concentrations when the second mechanism is working. Workers who support the

idea that both mechanisms are located at the plasmalemma (Welch and Epstein 1969) of course deny that the plasmalemma is freely permeable. Recent work (Nissen 1973a,b,c) has challenged the two mechanism hypothesis altogether and suggests there is one, multiphasic mechanism, located at the plasmalemma, which changes its kinetic properties as ionic availabilities differ. This model does not require, nor totally exclude, ion diffusion across the plasmalemma at high external nutrient concentrations. As discussed earlier (p 107), once water and ions have entered the root, either actively or passively, they have relatively free movement across the root until they reach the stele.

In the work reported here, several aspects of the uptake of heavy metals at the root surface were studied. No attempts were made to distinguish root uptake to outer space (passive) or to inner space (active). But since at least half, and often much more (see data of Papers 1 and 2), of the total metal of seedlings was found in shoots, most of the metals assayed in these experiments must have entered inner space before either transport to shoots or use for root metabolism. Even so, while the bulk of the effects would have been on metal movement into inner space, the movement into outer space may have been an appreciable component which should, in more precise studies, be considered.

The results reported in Paper 1 showed firstly that increasing the pH of the nutrient solution from 4 to 7 reduced uptake of Cu, Zn and Fe and had no effect on Mn, NO_3^-

or $HPO_4^=$ uptake. As the discussion (p 46) showed, the effects of pH on the actual uptake mechanisms of both cations and anions are poorly understood. Certainly uptake into both free space (Robertson 1958) and across membranes (Pitman 1970, Olsen 1953, Jackson and Adams 1963) are likely to be affected. Rubenstein (1974) suggests that H^+ ions may be involved in triggering the metabolic processes of active transport necessary for the uptake of Cl^- in Avena coleoptiles. The recent findings that auxin (Rubenstein 1974, Marre et al 1974b) and cytokinins (Marre et al 1974a) promote extrusion of H ions from cells which may then affect nutrient uptake, suggests that control of ion uptake at the root surface may be due to pH effects which are in turn under hormonal control. The results from this work gave little information on these matters. It appeared that P. radiata was more subject to root physiological damage as pH rose from 4-7 than a number of other higher plants; this may have led to the effects on nutrient uptake observed.

The second aspect of the factors which affect root ion uptake examined here, was the effects of competition between the heavy metals for uptake at the root surface. There has been fairly uncritical acceptance in the literature (p 44,78) that heavy metals compete with each other for uptake by roots. A few authors (Laties 1969, Schmid et al 1965) have noted that, in particular, very high concentrations of competing ions have often been used in uptake studies. The present theories of dual active uptake mechanisms (based on the work of Epstein and Hagen (1952)

with alkali cations) suggest that competition for uptake between ions would only occur when the second, non-specific, mechanism was operating when higher levels of ions were available.

A few authors have carried out experiments with heavy metals at moderate to low external metal concentrations. Two groups of authors have shown that with adequate, but not excessive external solution nutrient concentrations, there may be competition for uptake between certain heavy metals consistent with a non-specific mechanism operating at higher nutrient availabilities. Firstly, Schmid et al (1965) showed competitive inhibition of Zn uptake by Cu over a 30min period in excised barley roots. Zn was supplieed at about 0.3ppm and Cu at 0.1ppm. The second group (Malavolta et al 1956) used a long term (eight week) period for their experiment with coffee plants. Laties (1969) has pointed out that the kinetics of ion uptake may change over a few hours, so long term experiments mean little in attempting to closely define the relationships between ion uptake mechanisms. Nevertheless, Malavolta et al showed that Zn uptake from an 0.05ppm Zn solution was substantially reduced by 5ppm Mn and 0.2ppm Cu but not by 10ppm Fe. Another group of workers (Dokiya et al 1968) found some competition and some promotion between Cu, Mn and Fe being absorbed by barley and rice roots over a two hour period. The external concentrations of elements in these experiments did not rise above 0.1ppm and went down to well below sufficiency levels. The variability of the effects they

observed in their results, both between the two species and the different metals, does not suggest there were independent uptake mechanisms for all metals operating at low external metal concentrations.

The results in this work (Papers 1,2) gave no suggestion that there was marked competition between these elements for uptake by roots in the long term. Altering Zn and Cu concentrations in solution from below deficiency levels (deficiency below about 0.05ppm) to excess (1ppm) had no effects on Fe or Mn uptake when both were supplied at well above sufficiency levels (Fe at 5ppm and Mn at 3ppm) over 13-23weeks. There were some slight, but inconsistent, effects of Cu on Zn uptake but no reverse effects. The results do not support the concept that a non-specific uptake mechanism operates for these ions when present at above adequate concentrations. This assumes, of course, that the non-specific uptake mechanism would operate anywhere above adequate nutrient levels. This is not necessarily so: if this mechanism did not operate till very high metal concentrations in the external solution, then this may explain why other workers, using much higher concentrations, have observed competition for uptake between these metals. Considerable research is necessary to find definitive evidence of the existence or otherwise of similar uptake mechanisms for heavy metals as have been demonstrated for alkali cations.

Although the results showed little evidence of

competition for root uptake between the metals, results of Paper 5 showed that the rates of uptake of all four metals correlated moderately, but quite significantly. On the other hand, no significant correlations were found in Paper 4. The problems of interpretation of long term experiments such as these were discussed (p 134). There was no apparent cause for the differences between the results of the two experiments. If the results of Paper 5 are accepted, they suggest that the uptake mechanisms for these metals at the root surface are related, but the lack of competitive effects, discussed above, suggests the mechanisms were not the same. How these mechanisms might be related, but separate, is open to speculation. One common bond between attempts to isolate the carrier mechanisms involved in active transport across cell membranes is phosphorylation which generally seems to be involved at some stage of the process (Price 1970). Thus it may be that metals have different uptake mechanisms that vary their rates depending on the phosphorylative ability of the cells.

Nutrient transport across the root

A brief summary was given (p 107) of the path of transport of nutrient ions from the site of uptake at the root surface, across the root, to the stele. The transport may be either through the cell walls, for ions taken up passively, or through the cytoplasm via plasmodesmatal connections, for ions taken up actively. The endodermal

barrier stops the direct flow into the xylem of ions taken up passively.

The contention that ions must cross cell membranes at least twice, and therefore be subject to active control by the plant at least twice, before they are released to the xylem was generally supported in this work. Often there were very low correlations between the rates at which Cu, Zn, Mn and Fe were taken up by roots and the rates at which they were transported to shoots (Papers 4,5). This suggests that the root uptake and xylem release processes were independent, i.e. that there were two separate sites of control in the root. However, some significant correlations were observed, particularly between Cu, Zn and Mn. Further work is necessary to elucidate the meaning of these rather variable results.

Nutrient storage in the root

It was clear (Papers 1,2), that when any of the four metals studied were present in adequate supply in nutrient solutions, their concentration in roots exceeded that in shoots. The effect became more and more pronounced as the external nutrient concentration increased. This suggested that excess metal taken up by roots was stored in roots and not distributed throughout the seedling. Similar effects have been observed by other workers with higher plants (Hawf and Schmid 1967, Lopez and Graham 1973, Smith and Specht 1953, Hill 1973) but rarely have they been interpreted in the

same way as in this study.

Initially it was not clear if the storage of the excess metal was merely a defence mechanism against the high external nutrient concentration or a safety mechanism to store excess nutrient against a possible reduction in nutrient availability. The subsequent evidence suggested that both might apply. When seedlings were placed in Cu or Zn deficient solutions, after pretreatment with high Cu or Zn levels respectively, the stored metal was released from roots for transport to shoots (Paper 4). There was strong evidence that this was so for Cu and it appeared to be also true for Zn.

A corollary to these findings (Papers 2,3) was the indication that a large proportion of these heavy metals was associated with protein fractions in the root. If the metal storage were merely a defence mecanism, then the cell wall would seem the logical place to store the excess metal as does occur with Zn and Cu in grass clones especially tolerant of high levels of these elements (Turner 1970). There was evidence (Paper 2) that some Fe, which accumulated at very high concentrations in roots, may have been deposited in the cell wall. It seemed that the metals associated with protein were bound quite firmly within the protein structure and not merely at the protein surface, as they were not released by treatment with acid or alkali (Paper 3). The literature reviewed in Paper 4 suggested that there may be rapid protein breakdown and turnover in plants as a result of changing

plant physiological conditions. This suggests that even though excess metal may be held firmly within the protein structure, it can be quickly made available when required by the plant. It seemed unlikely that the protein that stored the excess metal was specifically synthesized for this purpose since changing the Zn or Cu concentration in the external solution had little effect on protein quantity in seedlings (Paper 2). It seemed likely that the fractions extracted from seedling tissue with water or tri-chloro acetic acid contained the protein which stored the excess metal (Paper 2).

These results provided substantial preliminary evidence for a root-protein metal storage system. However, some further work is necessary to confirm and expand on these findings. Firstly, support for the mechanism would be considerably increased if it could be shown that specific tissues contain the stored metal. Smith (1953) showed that both Cu and Zn accumulated in the hypodermis and particularly the endodermis of roots of sweet orange seedlings when these had been supplied with 10ppm of these elements for one hour. Substantial, but totally unsuccessful, attempts were made here to find what tissues stored Zn and Cu in roots of P. radiata seedlings. At first, chemical stains specific for these metals were tested. Root segments of seedlings grown for several weeks in nutrient solutions with excess Zn or Cu were fixed, dehydrated and mounted in glycol methacrylate by the technique of Feder and O'Brien (1968) before sectioning. For Zn, Mayer et al's Zn-dithizone staining technique was

used (McManus and Mawry, 1960) and for Cu, Mallory and Parkin's haematoxylin stain was tested (Glick 1949). No meaningful staining occurred with either stain. Nor was there any result when frozen sections, prepared by the method of Knox (1970) and cut on a freezing microtome, were used instead of the glycol methacrylate mounted sections.

After these unsuccessful attempts, sections were prepared for metal assay with an X-ray micro-probe in a Cambridge scanning electron microscope. To improve the contrast in the microscope, attempts were made to etch the glycol methacrylate away from the sections (1μm thick) using a saturated solution of NaOH in ethanol (Thurley and Mouel 1974). This proved unsuccessful, but when Spurr's (1968) medium replaced the glycol methacrylate, thorough etching was achieved. Nevertheless, the X-ray probe was unable to detect appreciable quantities of Cu, Zn, Mn or Fe in the sections. Even when a freshly cut piece of root was placed on the microscope stage, no appreciable quantities of heavy metals could be detected. Further work, perhaps with other metal stains would seem worthwhile to pursue this matter.

Further work is also necessary to verify that excess metals are held in association with protein. This would necessitate purification of the protein involved and tests to determine that these metals may be readily included in its structure.

Release of metals from roots and transport to shoots

The second site for control of ion uptake by plants is at the endodermal barrier in the root (p 107). It has been proposed that the xylem parenchyma cells which are rich in cytoplasm and mitochondria may be the cells which actively secrete ions into the xylem stream (Lauchli et al 1971, 1974). In this work, the rates at which Cu, Zn, Mn and Fe moved from roots to shoots correlated very highly (Paper 4) or moderately (Paper 5) as long as metal supply in the nutrient solution was adequate. This supports the suggestion that the mechanisms controlling release of metals to the xylem are related, just as they were for metal uptake at the root surface. It is thought that the active transport mechanisms at the endodermis are the same as those at the epidermis (Dunlop 1974). It has generally been assumed that the active processes operate from within the cell to the xylem, but Dunlop (1974) suggested that the process might act in the reverse direction. The direction of active transport at the epidermis has also been questioned by Higinbotham et al (1967) who suggested that for some cations active transport may operate from within the cell to the outside rather than the reverse. In either case, passive ion movement may occur in the opposite direction (Dunlop 1974). The direction of the active steps controlling ion transport in plants would not alter the arguments suggesting that the transport mechanisms may be related for the different metals.

As with uptake by roots, there was very little

suggestion of substantial competition between the ions considered here for transport to shoots (Papers 1,2) even when metals were in above sufficient supply. This again contravenes current theories for active ion transport mechanisms.

Once in the xylem, nutrient ions move passively up the stem in association with the transpiration stream. Often the rates of ion movement and rate of water flow have been closely correlated. The controversy surrounding the interpretation of these observations was discussed in Paper 4 (p 108,136). The results of Paper 4 showed no significant correlations relating rate of Cu, Zn, Mn or Fe movement to shoots and transpiration. It was suggested (p 137) that at least Cu, Zn and Mn may move up the xylem by a series of exchange reactions with exchange sites on the walls of the xylem. This has been observed elsewhere with Ca, Sr and Zn. Fe, on the other hand, because it is readily oxidised from Fe^{2+} to Fe^{3+} and the hydroxide of the trivalent form is highly insoluble, is generally found chelated with organic substances in the xylem. The availability and electrical properties of chelates may affect the transport of Fe in the xylem stream and prevent correlation with the rate of flow of the transpiration stream. The problem of interpretation of these results in long term experiments was also discussed (p 136).

The fate of inorganic ions on reaching the leaves depends largely on their metabolic function. Thus, Cu and Fe

for example, both of which have roles in photosynthesis, may be localised in chloroplasts. Possible control mechanisms for the distribution of ions to the various parts of the plant crown are discussed briefly in the final section of these conclusions.

Heavy metal metabolism in the plant

Discussion to this point has been concerned with the entry of heavy metals into plants and the control of their distribution between roots and shoots. These matters were the major considerations of this work. But inevitably, as they were pursued, some observations were made of the metabolic effects of the metals. The discussion here will briefly review the biochemical roles of heavy metals in plants and relate the few relevant observations made in these experiments to these roles.

The essentiality of a group of mineral elements for the growth and completion of the life cycles of higher plants is beyond doubt. Hewitt (1963) has concluded that N, P, K, Ca, Mg, S, Cu, Zn, Mn, Fe, Mo, B, Cl are essential to higher plants, while Na, Co, Se, Al, Ge, F may be beneficial to growth but not essential. Price (1970) and Nason and McElroy (1963) add V to the list of essential elements. Marked growth reductions with Zn and Cu deficiency, and symptoms of Zn deficiency similar to those reported elsewhere (Smith and Bayliss 1942), were observed in this work (Frontpiece, Paper 1). Critical levels required for maximum growth of

seedlings both in the nutrient solution and in roots and shoots were determined. The results agreed generally with data for other conifers - no similar data for P. radiata seedlings at an early age could be located.

For the essential micronutrients (Cu, Zn, Mn, Fe, Mo, B, Cl, V), the major role in plant metabolism seems to be as co-factors or activators in enzyme systems, although no direct evidence exists for this in the cases of B and Cl (Nason and McElroy 1963). Nason and McElroy have broadly reviewed the topic of micronutrient metabolism, and their review forms the basis of the discussion below.

Two groups of enzymes which require heavy metals for their activity can be recognised. In the first group, the protein contains a specific metal as an integral component of its structure. Metals bound in this way may alter the electrical properties of the protein. This may, in turn, alter the ability of an enzyme to bind to its substrate or its activity under different environmental conditions, e.g. with changes in pH. These metals may also be important in maintaining the physical structure of proteins. Thus Zn maintains the quaternary structure of alcohol dehydrogenase in yeast (Kogi and Vallee 1960). It has also been suggested that the conformation of nucleic acids may be stabilized by heavy metals which are commonly found in nucleic acid isolates (Wacker and Vallee 1959, Fuwa et al 1960). Of the heavy metals, Zn, Cu, Fe and Mo are all co-factors to this group of enzymes. The second enzyme group uses metals as

activators to increase catalytic activity. The metal may serve as a bridge between the protein and its substrate or it may stabilize the structure of an intermediate in the protein-substrate reaction. Mn, V and possibly B are thought to act in this way. Some brief notes on the specific roles of the four metals examined in this study (Cu, Zn, Mn, Fe) are given below.

The major groups of enzymes with which Cu is involved are the phenolases, laccase and oxidases (Nason and McElroy 1963, Hill 1973) and is particularly involved with photosynthesis (Hill 1973, Cedeno-Maldonado et al 1972) probably in the electron transport process (Kato and Takamiya 1961): much leaf Cu is found associated with chloroplasts (Neish 1939, Kato et al 1961). A substantial photosynthetic role for Cu would imply a higher leaf Cu requirement than root, contrary to the conclusions of Paper 1 where roots required a higher Cu concentration than shoots for maximum growth. Doubts as to the interpretation of these results were discussed (p 43). Cu may also have a role in chlorophyll formation (Nason and McElroy 1963). No evidence for this was found in this study where Cu deficiency had no effect on photosynthetic pigment levels (Paper 1).

The role of Zn as a co-factor to enzymes in IAA synthesis and degradation has already been discussed (Paper 5). Zn also appears to act as a co-factor to a phosphate transferring enzyme (Nason and McElroy 1963) and the effects Zn deficiency may therefore have on protein

synthesis were discussed (p 77). Other enzymes, notably dehydrogenases and carbonic anhydrase, are Zn requiring enzymes (Price 1970, Nason and McElroy 1963). Zn deficiency reduced synthesis of photosynthetic pigments in this work, but it was concluded that this was a secondary effect of the deficiency (Paper 1).

The terminal respiratory chain which transfers electrons from substrates to oxygen is mediated by the cytochromes which contain Fe-porphyrin moieties and involves the oxidation-reduction of Fe^{2+} and Fe^{3+} (Nason and McElroy 1963). This is perhaps the most important role of Fe in plants. Fe also appears to be important in the structural development of chloroplasts (Price 1968,1970) A summary of the numerous metabolic lesions of Fe deficiency and the substances with which Fe is associated in the plant is given by Price (1968).

As mentioned above, Mn does not form part of the structure of Mn-requiring enzymes, but acts rather as an activator to greatly increase their catalytic activity. Nason and McElroy (1963) give a long list of enzymes which are activated by Mn, many of which have been found in higher plants. These cover a broad range of plant metabolic activities. Because there is no specific structural requirement for Mn in enzymes, it can often be replaced by other metals (notably Mg) which have similar effects on the catalytic activity of the enzymes. Mn deficiency often leads to a severe reduction in rate of photosynthesis although

chlorophyll levels are unaffected; the mechanism of this effect is unknown. A role for Mn in catalyzing the oxidative decarboxylation of IAA has been well established (MacLachlan and Waygood 1956, Waygood et al 1956). It seems surprising then that Skoog (1940) (see p 160) observed no effect of Mn deficiency on IAA levels in tomato until after the plant metabolism had been severely affected.

Some specific metabolic processes which may have been affected by heavy metals were briefly studied in this work. Observations were made of the effects of Zn and Cu deficiencies on N compounds in seedlings (Paper 2). There was general agreement with other work that these deficiencies caused some changes in protein quality but not total quantity. Both Zn and Cu deficiencies increased the amount of total soluble amino acids in shoots as has been observed elsewhere (Steinberg 1956, Possingham 1956) but not in roots. Attempts were made here to determine which amino acids were affected by metal deficiencies. The constituents of the water soluble fraction extracted from P. radiata tissue (see Paper 3) were separated by electrophoresis using the method of Rothman and Higa (1962) in a pH 2 acetic-formic acid buffer. Electrophoretograms were developed for amino acids with an 0.5% ninhydrin spray. Other compounds in the extracts confused the separation of the amino acids and no qualitative differences were apparent. Two-way paper chromatograms, using first a butanol-acetic acid-water solvent then a phenol-ammonia solvent (Smith 1965) did not improve the results. Attempts to purify the extracts by passing them

through an H^+ exchange column (Dowex-50) and eluting amino acids with ammonia did not remove interfering substances sufficiently to allow any meaningful separation of amino acids. It appeared that extensive analytical work would be necessary to find suitable procedures to purify P. radiata extracts to allow assay of individual amino acids.

Wacker (1962) working with the unicellular flagellate Euglena gracilis, suggests that the primary metabolic lesion in Zn deficiency is the derangement of RNA structure which requires Zn for its maintenance. He proposes that this leads to decreased protein synthesis which may then cause a build up of free amino acids similar to that observed in this work. An amino acid build up need not necessarily signal a decline in protein synthesis. Both amino acids and proteins may be synthesized or degraded in a number of systems any or all of which may be affected by Zn deficiency in the long or short term. Also, a build up of amino acids in both shoots and roots would be expected if protein was degraded, not only in shoots as observed here. Nason and McElroy (1963) propose rather that Zn may be involved in the synthesis of larger and more complex protein molecules some time after the primary stage of protein synthesis. But the synthesis of small proteins is unaffected by the Zn deficiency. The levels of some proteins have been found to increase in Zn deficient Neurospora (Nason et al 2951) verifying that not all protein synthesis was reduced by Zn deficiency. There seems relatively little discussion in the literature on the interpretation of the effects of Cu

deficiency on amino acid levels which were similar to those observed for Zn.

Neither Zn nor Cu deficiency had significant effects on the level of soluble sugars in shoots or roots (Paper 2). It was suggested that the deficiencies induced here were not sufficiently severe, or did not persist long enough, to reudce photosynthesis as might ultimately be expected in any plant of which the metabolism was severely disturbed. This seems especially surprising in the case of Cu deficiency, as Cu is directly involved with photosynthesis (see above). It was concluded in Paper 2 that the decline in growth rate with metal deficiency was primarily due to reduced activity of metal-requiring enzymes. The later conclusions of Paper 5 suggested that, for Zn deficiency at least, growth may decline, at least initially, due to reduced auxin availability in the plant.

Hormonal control of plant nutrition

The involvement of plant hormones in a vast range of different aspects of plant life (Thimann 1963, Price 1970) suggests that plant nutrition may be hormone controlled just as much as other physiological processes. Yet work on the effects of hormones on the control of nutrition has gained momentum only in the last five years as the literature reviewed in the Introduction to Paper 5 makes clear.

The miscellany of effects that plant hormones

produce, and our continuing lack of understanding of their mechanism of action, makes the interpretation of any result in the light of an overall theory of hormone action impossible (Thimann 1963). This problem occurred in interpreting the results of Paper 5 where a hypothesis for a control mechanism of Zn nutrition by the plant was proposed. This hypothesis was based on observations from other work that Zn deficiency reduces the rate of auxin synthesis in the plant and increases its rate of breakdown. This then reduces auxin stimulated growth so that the plant does not grow vigorously when an essential element is deficient. The results of this work suggested that, in addition, the root Zn uptake mechanism is stimulated by the reduced auxin level so the plant has maximum ability to absorb any Zn available in the external solution. How such a system fits into the overall system of plant metabolism is not known.

Effects of IAA, GA and ABA on Cu and Mn transport to shoots, as well as the effects on Zn uptake by roots, were also observed in this work. Discussion in Paper 5 pointed out the difficulty of relating these observations to the natural plant system when hormones are applied exogenously to the plant at relatively arbitrary concentrations. Jenkins (1971) and Shepherd (1965) have observed auxin-, gibberellin-, cytokinin- and inhibitor-like activity in extracts from P. radiata tissue, so there seems little doubt that these substances are normally present in this species. Observation of the effects of metal deficiency on the endogenous levels of these hormones would seem the next step

In pursuing this line of study of control of plant nutrition.

There are a number of other areas where plant hormones might have a role in control of plant nutrition. Certain tissues, e.g. rapidly growing tissue, require a much larger supply of nutrients (organic and inorganic) than other tissues. Hormones may determine to which tissues nutrients coming from roots are directed. Inorganic nutrients may be mobilized from senescing leaves and transported elsewhere for use: this process too might be hormone controlled. The release of excess metal stored in roots for use by shoots, as discussed earlier in this work, must also occur in response to a signal which may be hormonal. These possibilities offer extensive opportunities for further research.

BIBLIOGRAPHY

Addicot, F.T. 1972 Biochemical aspects of the action of abscisic acid. p272-80 In Carr, D.J. (Ed) Plant Growth Substances 1970. Springer-Verlag, Berlin, Heidleberg, N.Y.

———— and J.L. Lyon 1969 Physiology of abscisic acid and related substances. Ann. Rev. Pl. Physiol 20: 139-64

Anon 1971 Determination of ammonia in waste water. Technicon Industrial Systems, Industrial Method 18-69W

Arnon, D.I. 1949 Copper enzymes in isolated chloroplasts. Polyphenol oxidase in Beta vulgaris. Pl. Physiol 24: 1-5

————, W.E. Fratzke and C.M. Johnson 1942 Hydrogen ion concentration in relation to absorption of inorganic nutrients of higher plants. Pl. Physiol 17: 515-24

———— and C.M. Johnson 1942 Influence of hydrogen ion concentration on growth of higher plants under controlled conditions. Pl. Physiol 17: 525-39

Asher, C.J., P.G. Ozanne and J.F. Loneragan 1965 A method for controlling the ionic environment of plant roots. Soil Sci 100:149-56

Ashour, N.I., U. Zur Nieder and E. Mueller 1974 The action of (2-chloro-ethyl)-trimethyl-ammonium chloride (CCC) and giberellic acid (GA) on uptake and distribution of ^{45}Ca in tall and dwarf pea plants. Biochem. Physiol. Pflanz 165: 497-504

Barker, P.R. 1973 Nutrition of Pinus radiata D.Don with particular reference to fine root production and phosphorus-zinc relationships. PhD Thesis, A.N.U., Canberra

Beardsell, M.F. and D. Cohen 1974 Endogenous abscisic acid-plant water stress relationships under controlled environmental conditions. p411-5 In Bieleski, R.L., A.R. Ferguson and M.M. Cresswell (Eds) Mecanisms of Regulation of Plant Growth. Bull 12, Royal Soc., N.Z.

Bell, C.W. and O. Biddulph 1963 Translocation of calcium. Exchange versus mass flow. Pl. Physiol 38: 610-4

Bell, D.J. 1955 Mono- and oligo-saccharides and acidic monosaccharide derivatives. p1-54 in Tracey, M.V. and K. Paech (Eds) Modern Methods of Plant Analysis Vol II. Springer-Verlag, Berlin

Benzian, B. and R.G. Warner 1956 Copper deficiency in Sitka spruce seedlings. Nature 178: 864-5

Biddulph, A., F.S. Nakayama and R. Cary 1961 Transpiration and ascension of calcium. Pl. Physiol 36: 429-36

Biddulph, O. 1959 Translocation of inorganic solutes. p553-603 in Steward, F.C. (Ed) Plant Physiology. A Treatise Vol II. Acad. Press, N.Y.

Bollard, E.G. 1958 Nitrogenous compounds in tree xylem sap. p83-93 in Thimann, K.V. (Ed) The Physiology of Forest Trees. Ronald Press Co., N.Y.

Bowen, J.E. 1969 Absorption of copper, zinc and manganese by sugarcane leaf tissue. Pl. Physiol 44: 255-61

Brady, C.J. 1973 Changes accompanying growth and senescence and effect of physiological stress. p317-51 in Butler, G.W. and R.W. Bailey (Eds) Chemistry and Biochemistry of Herbage Vol II. Acad. Press, Lond., N.Y.

Briggs, G.E. and R.N. Robertson 1957 Apparent free space. Ann. Rev. Pl. Physiol 8: 11-30

Brouwer, R. 1965 Ion absorption and transport in plants. Ann. Rev. Pl. Physiol 16: 241-66

Brown, J.C. 1963 Interactions involving nutrient elements. Ann. Rev. Pl. Physiol 14: 93-106

Bukovac, M.J. and S.H. Wittwer 1957 Absorption and mobility of foliar applied nutrients. Pl. Physiol 32: 428-35

Burton, K. 1956 A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J 62: 315-23

Cedeno-Maldonado, A. and J.A. Swader 1972 The cupric ion as an inhibitor of photosynthetic electron transport in isolated chloroplasts. Pl. Physiol 50: 698-701

Cochran, W.G. and G.M. Cox 1957 Experimental Designs. Wiley 611pp

Collins, J.C. and A.P. Kerrigan 1973 Hormonal control of ion movement in the plant root? p589-93 in Anderson, W.P. (Ed) Ion Transport in Plants. Acad. Press, Lond., N.Y.

——————— and ——————— 1974 The effect of kinetin and abscisic acid on water and ion transport in isolated maize roots. New Phytol 73: 309-14

Cooley, W.W. and P.R. Lohnes 1962 Multivariate Procedures for the Behavioural Sciences. John Wiley & Sons Inc. 211pp

——————— and ——————— 1971 Multivariate Data Analysis John Wiley & Sons Inc. 364pp

Cram, W.J. and M.G. Pitman 1972 The action of abscisic acid on ion uptake and water flow in plant roots. Aust. J. Biol. Sci 25: 1125-32

Davies, C.R. and P.F. Wareing 1965 Auxin-directed transport of radiophosphorus in stems. Planta (Berl) 65: 139-56

Diez-Alteres, C. and E.Bornemisza 1967 The localisation of zinc-65 in germinating corn tissues. Pl. Soil 26: 175-88

Doerffling, K., V. Menzen and Anita Gerloehluessow 1973 Antagonistische Wirkungen von Abscisinsaeure einerseits und Indole-3-essig-saeure sowie Gibberellinsaeure anderseits auf den Transport von Kalium und Phosphor in Helianthus Epicotylen. Mitt. Staatsirt. Allg. Bot., Hamburg 14: 19-23

Dokiya, Y., N. Owa and S. Mitsui 1968 Comparitive physiological study of iron, manganese and copper absorption by plants. Soil. Sci. and Pl. Nut 14: 169-74

Dunlop, J. 1974 A model of ion translocation by roots which allows for regulation by physical influences. p133-8 in Bieleski, R.L., A.R. Ferguson and M.M. Cresswell (Eds) Mechanisms of Regulation of Plant Growth. Royal Soc. N.Z. Bull 12

Dunne, T.C. 1956 A zinc-copper antagonism affecting cereals. Aust. Pl. Nutrition Conf 1: 278-83

Emmert, F.H. 1965 Forces involved in retention and movement of non-metabolic strontium in Phaseolus vulgaris. Pl. Soil 22: 136-42

Epstein, E. 1966 Dual pattern of ion absorption by plant cells and by plants. Nature 212: 1324-7

Epstein, E. and C.E. Hagen 1952 A kinetic study of the absorption of alkali cations by barley roots. Pl. Physiol 27: 457-74

Feder, N. and T.P. O'Brien 1968 Plant microtechniques: some principles and new methods. Amer. J. Bot 55: 123-42

Fleck, A. and H.N. Munro 1962 The precision of ultraviolet absorption measurements in the Schmidt-Thannhauser procedure for nucleic acid estimation. Biochim. Biophys. Acta 55: 571-83

Fogg, D.N. and N.T. Wilkinson 1958 The colorimetric determination of phosphorus. Analyst Lond. 83: 406-14

Fuehring, H.D. and G.S. Soofi 1964 Nutrition of corn on a calcareous soil: II. Effect of zinc on the yields of grain and stover in relation to other micronutrients. Soil Sci. Soc. Amer. Proc 28: 79-82

Fuwa, K.W., W.E.C. Wacker, R. Oruyon, A.F. Bartholomay and B.L. Vallee 1960 Nucleic acids and metals. II. Transition metals as determinants of conformation of ribonucleic acids. Proc. Nat. Acad. Sci 46: 1298-1307

Gilbert, G.S. 1951 A biochemical basis for copper-nitrogen balance in tung. Pl. Physiol 26: 398-405

Glick, D. 1949 Techniques of Histo- and Cytochemistry. Intersc. Publishers Inc., N.Y., Lond.

Glinka, Z. and L. Reinhold 1971 Abscisic acid raises the permeability of plant cells to water. Pl. Physiol 48: 103-5

Greenwood, E.A.N. and E.G. Hallsworth 1960 Studies on the nutrition of forage legumes. II. Some interactions of calcium, phosphorus, copper and molybdenum on the growth and chemical composition of Trifolium subterraneum L. Pl. Soil 12: 97-127

Gurd, F.R.N. 1970 Binding of protons and other ions. p365-436 in Leach, S.J. (Ed) Physical Principles and Techniques of Protein Chemistry. Pt B. Acad. Press, N.Y.

Hall, M.J. 1961 The establishment of pines on poor deep sands at Longford Victoria. Appita 15: 68-76

Harborne, J.B. 1973 Flavonoids. p344-80 in Miller, L.P. (Ed) Phytochemistry. Vol II. Van Nostrand Reinhold Co.

Harley, J.L. 1952 Associations between microorganisms and higher plants (mycorrhiza). Ann. Rev. Microbiol 6: 367-86

———— 1969 Mycorrhiza and nutrient uptake in forest trees. p163-79 in Luckwill, L.C. and C.V. Cutting (Eds) Physiology of Tree Crops. Acad. Press, Lond., N.Y.

Haurowitz, F. 1963 The Chemistry and Function of Proteins. 2nd Ed., Acad. Press, N.Y. 455pp

Hawf, L.R. and W.E. Schmid 1967 Uptake and transportation of zinc by intact plants. Pl. Soil 27: 249-60

Helleburst, J.A. and R.G.S. Bidwell 1964 Protein turnover in attached wheat and tobacco leaves. Can. J. Bot 42: 1-12

Hewitt, E.J. 1963 The essential nutrient elements: requirements and interactions. p137-360 in Steward, F.C. (Ed) Plant Physiology. A Treatise. Vol III. Acad. Press, N.Y.

———— 1966 Sand and Water Culture Methods used in the Study of Plant Nutrition. Comm. Ag. Bureaux, Tech. Comm. No 22, 547pp

Hewitt, W.M. and M.E. Gardner 1956 Some studies of the adsorption of zinc sulphate in Thompson seedless grape canes. Pl. Physiol 31: 393-9

Higinbotham, N., B. Etherton and R.J. Foster 1967 Mineral ion contents and cell transmembrane electropotentials of pea and oat seeling tissue. Pl. Physiol 42: 37-46

Hill, J.M. 1973 The change with age in the distribution of copper and some copper-containing oxidases in red clover (Trifolium pratense L. cv. Dorset Marl-grass). J. Exp. Bot 24: 525-36

Hoagland, D.R. and T.C. Broyer 1937 Hydrogen-ion effects and the accumulation of salt by barley roots as influenced by metabolism. Amer. J. Bot 27: 173-85

Hodgson, J.F. 1963 Chemistry of the micronutrient elements in soils. Adv. Agron 15: 119-59

Honert van den, T.H. and J.J.M. Hooymans 1955 On the absorption of nitrate by maize in water culture. Acta Botanica Neerlandica 4: 376-84

Hope, K. 1968 Methods of Multivariate Analysis. Univ. Lond. Press Ltd. 288pp

Huber, W. and N. Sankla 1974 Abscisic acid: kinetin interaction in growth and activities of enzymes of amino-acid metabolism in Pennisetum typhoides seedlings. Z. Pflanzenphysiol 73: 160-6

Hughes, D.W. and K. Genest 1973 Alkaloids. p118-70 in Miller, L.P. (Ed) Phytochemistry. Vol II. Van Nostrand Reinhold Co.

Hurd, R.G. and J.F. Sutcliffe 1957 An effect of pH on the uptake of salt by plant cells. Nature 180: 233-5

Hutchinson, W.C. and H.N. Munro 1961 The determination of nucleic acids in biological materials. Analyst 86: 768-813

Ishizuka, Y. and T. Ando 1968 Interaction between manganese and zinc in growth of rice plant. Soil Sci. and Pl. Nut 14: 201-6

Jackson, M.L. 1958 Soil Chemical Analysis. Constable and Co., Ltd., Lond. 498pp

Jackson, P.C. and H.R. Adams 1963 Cation-anion balance during potassium and sodium absorption by barley roots. J. Gen. Physiol 46: 369-86

Jacobsen, L., R. Overstreet, R.M. Carlson and J.A. Chastain 1957 The effect of pH and temperature on the absorption of potassium and bromide by barley roots. Pl. Physiol 32: 658-62

Jaspars, E.M.J. 1956 Pigmentation of tobacco crown-gall tissues cultured in vitro in dependence of the composition of the medium. Physiol. Plant 18: 933-40

Jenkins, P.A. 1971 Growth substances and cambial activity in radiata pine. PhD Thesis, A.N.U., Canberra

Jennings, D.H. 1963 The Absorption of Solutes by Plant Cells. Oliver and Boyd, Edin., Lond. 204pp

Johnson, W.M.J. and W.G. Schrenk 1963 Zinc distribution in alfalfa plant cell. Trans. Kansas Acad. Sci 66: 429-34

Kannan, S. and T. Mathew 1970 Effects of growth substances on the absorption and transport of iron in plants. Pl. Physiol 45: 206-9

Kato, S. and A. Takamiya 1961 A new leaf copper protein 'plastocyanin', a natural Hill oxidant. Nature (Lond.) 189: 665-6

————, I. Suga, I. Shiratori and A. Takamiya 1961 Distribution of plastocyanin in plants with special reference to its localization in chloroplasts. Arch. Biochem. Biophys. 94: 136-41

Keller, S. and R.J. Block 1960 Separation of proteins. p2-30 in Keller, S. and R.J. Block (Eds) A Laboratory Manual of Analytical Methods of Protein Chemistry. Vol I. Pergamon Press, Lond., N.Y., Paris

Knox, R.B. 1970 Freeze sectioning of plant tissues. Stain Tech 45: 265-72

Kogl, J.H. and B.L. Vallee 1960 The role of zinc in alcohol dehydrogenase. V. The effect of metal-binding agents on the structure of the yeast alcohol dehydrogenase molecule. J. Biol. Chem 235: 3188-92

Kositsyn, A.V. and T.I. Iyoshina 1964 Vrutnikletochre respredelerie v listorols tkani tomatov. Fiziologya Rost 11: 175-80 (English Summary)

Kramer, P.J. 1959 Transpiration and the water economy of plants. p607-726 in Steward, F.C. (Ed) Plant Physiology. A Treatise. Vol III. Acad. Press, N.Y.

———— 1969 Plant and Soil Water Relationships. McGraw Hill Book Co. 428pp

Kriedeman, P.E. and B.R. Loregs 1974 Hormonal mediation of plant responses to environmental stress. p461-5 in Bielski, R.L., A.R. Ferguson and M.M. Cresswell (Eds) Mechanisms of Regulation of Plant Growth. Bull 12 Royal Soc. N.Z.

Kuiper, F. and P.J.C. Kuiper 1974 Permeability and self induction as factors in water transport through bean roots. Physiol. Plant 31: 159-62

Laties, G.C. 1969 Dual mechanisms of salt uptake in relation to compartmentation and long-distance transport. Ann. Rev. Pl. Physiol 20: 89-110

Lauchli, A. 1972 Translocation of inorganic solutes. Ann. Rev. Pl. Physiol 23: 197-218

————, D. Kramer, M.G. Pitman and U. Lueltge 1974 Ultrastructure of xylem parenchyma cells of barley roots in relation to ion transport in the xylem. Planta 119: 85-99

Lauchli, A., A.R. Spurr and E. Epstein 1971 Lateral transport of ions into the xylem of corn roots. II. Evaluation of a stelar pump. Pl. Physiol 48:118-24

Li, T.K. 1966 The functional role of zinc in metalloenzymes. p46-48 In Prasad, A.S. (Ed) Zinc Metabolism. Charles C. Thomas, Springfield, Illinois

Lingle, J.C., L.D. Tiffin and J.C. Brown 1963 Iron uptake-transport of soybeans as influenced by other cations. Pl. Physiol 38: 71-6

Lopez, P.L. and E.R. Graham 1973 Labile pool and plant uptake of micro-nutrients: II. Uptake of Mn, Fe and Zn by Ladino clover (Trifolium repens) and its relation to soil labile pools. Soil Sci 115: 380-9

Lowry, O.H., N.J. Rosenbrough, A.L. Farr and R.J. Randall 1951 Protein measurement with the Folin phenol reagent. J. Biol. Chem 193: 265-75

McLachlan, G.A. and E.R. Waygood 1956 Kinetics of enzymatically catalyzed oxidation of indole acetic acid. Can. J. Biochem. Physiol 34: 1233-50

McManus, J.F.A. and R.W. Mawry 1960 Staining Methods Histologic and Histochemical. Paul B. Hoeber, N.Y. 423pp.

Malavolta, E., J.P. Anzolla and H.P. Haag 1956 Preliminary note on the absorption of radiozinc by young coffee plants (Coffea arabica L.) grown in nutrient solution. Phyton 6: 1-6

Mansfield, T.A. and R.J. Jones 1971 Effects of abscisic acid on potassium uptake and starch content of stomatal guard cells. Planta (Berl) 101: 147-58

Marre, E., R. Colombo, P. Lado and F. Rasi-Caldogno 1974a Correlation between proton extrusion and stimulation of cell enlargement: effects of cytokinins on leaf fragments and isolated cotyledons. Pl. Sci. Lett 2: 139-50

———, P. Lado, A. Ferrari and A. Ballarin Denti 1974b Transmembrane potential increase induced by auxin, benzyladenine and fusococcin: correlation with proton extrusion and cell enlargement. Pl. Sci. Lett 2: 257-65

Marschner, H. 1974 Mechanisms of regulation of mineral nutrition in higher plants. p99-109 In Bielski, R.L., A.R. Ferguson and M.M. Cresswell (Eds) Royal Soc. N.Z. Bull 12

Martin, P.G. 1966 Variation in the amounts of nucleic acids in cells of different species of higher plants. Exp. Cell Res 44: 84-94

Melin, E., H. Nilsson and E. Hacskaylo 1958 Translocation of cations to seedlings of Pinus virginiana through mycorrhizal mycelium. Bot. Gaz 119: 243-6

Millikan, C.R. 1953 Relative effects of zinc and copper deficiencies on lucerne and subterraneum clover. Aust. J. Biol. Sci 6: 164-77

Milthorpe, F.L. and J. Moorley 1969 Vascular transport and its significance in growth. Ann. Rev. Pl. Physiol 20: 117-38

Molz, F.J. and E.I. Ikenberry 1974 Water transport through plant cells and cell walls: theoretical development. Soil Sci. Soc. Amer. Proc 38: 699-704

Most, B.H. 1971 Abscisic acid in immature apical tissue of sugar cane and in leaves of plants subjected to drought. Planta (Berl) 101: 67-75

Munns, D.N. and C.M. Johnson 1960 Removal of heavy metal and halide contamination from macronutrient salts. Pl. Physiol 35: 978-81

Nason, A. 1952 Metabolism of micronutrient elements in higher plants. II. Effect of copper deficiency on the isocitric enzyme in tomato leaves. J. Biol. Chem 198: 643-53

——— and W.D. McElroy 1963 Modes of action of the essential mineral elements. p451-536 in Steward, F.C. (Ed) Plant Physiology. A Treatise. Vol III. Acad. Press, N.Y.

———, H.A. Oldewurtel and L.M. Propst 1952 Role of micronutrient elements in the metabolism of higher plants. I. Changes in oxidative enzyme constitution of tomato leaves deficient in micronutrient elements. Arch. Biochem. Biophys 38: 1-13

Neish, A.C. 1939 Studies on chloroplasts. II. Their chemical composition and the distribution of certain metabolites between the chloroplasts and the remainder of the leaf. Biochem. J 33: 300-8

Nissen, P. 1973a Kinetics of ion uptake in higher plants. Physiol. Plant 28: 113-20

——— 1973b Multiphasic uptake in plants. I. Phosphate and sulfate. Physiol. Plant 28: 304-16

Nissen, P. 1973c Multiphasic ion uptake in roots. p539-53 in Anderson, W.P. (Ed) Ion Transport in Plants. Acad. Press, Lond., N.Y.

Oelke, E.A. and R.H. Andrew 1966 Chlorophyll relationships for certain sweet corn genotypes in different environments. Crop Sci 6: 113-6

Ogur, M. and G. Rosen 1950 The nucleic acids of plant tissues. I. The extraction and estimation of desoxypentose nucleic acid and pentose nucleic acid. Arch. Biochem 25: 262-76

Oldenkamp, L. and K.W. Smilde 1966 Copper deficiency in Douglas Fir (Pseudotsuga menziesii (Mirb.) Franco). Pl. Soil 25: 150-2

Olsen, C. 1953 The significance of concentration for the rate of ion absorption by higher plants in water culture. IV The influence of hydrogen ion concentration. Physiol. Plant 6: 848-58

Ozanne, P.G. 1955 The effect of nitrogen on zinc deficiency in subterraneum clover. Aust. J. Biol. Sci 8: 47-55

Paleg, L.R. 1965 Physiological effects of gibberellins. Ann. Rev. Pl. Physiol 16: 291-322

Penny, M.G. and D.J.F. Bowling 1974 A study of potassium gradients in the epidermis of intact leaves of Commelina communis L. in relation to stomatal opening. Planta (Berl) 119: 17-25

Pirie, N.W. ,1973 p112-3 In Rothamstead Experimental Station Ann. Report 1972, Pt 1

Pitman, M.G. 1970 Active H^+ efflux from cells of low-salt barley roots during salt accumulation. Pl. Physiol 45: 787-90

Polson, D. and M.W. Adams 1970 Differential response of navy beans (Phaseolus vulgaris L.) to zinc. I. Differential growth and elemental composition at excessive zinc levels. Agron. J 62: 557-60

Possingham, J.V. 1956 The effect of mineral nutrition on the content of free amino acids and amides in tomato plants. I. A comparison of the effects of copper, zinc, manganese, iron and molybdenum. Aust. J. Biol. Sci 9: 539-51

Price, C.A. 1968 Iron compounds and plant nutrition. Ann. Rev. Pl. Physiol 19: 239-48

Price, C.A. 1970 Molecular Approaches to Plant Physiology. McGraw Hill Book Co. 398pp

———— and B.L. Vallee 1962 Euglena gracilis, a test organism for study of zinc. Pl. Physiol 37: 428-33

Racusen, D. and M. Foote 1962 Protein turnover rate in bean leaf discs. Pl. Physiol 37: 640-2

Ricklefs, R.E. 1967 A graphical method of fitting equations to growth curves. Ecol 48: 978-83

Robertson, R.N. 1958 The uptake of minerals. p243-79 in Ruhland, W. (Ed) Encyclopaedia of Plant Physiology. Vol IV. Springer-Verlag, Berlin

Rosen, H. 1957 A modified ninhydrin colorimetric analysis for amino acids. Arch. Biochem. Biophys 67: 10-15

Rothman, F. and A. Higa 1962 A new two-dimensional system for the separation of amino acids on paper. Analytical Biochem 3: 173-7

Routien, J.B. and R.F. Dawson 1943 Some interrelationships of growth, salt absorption respiration and mycorrhizal development in Pinus echinata Mill. Amer. J. Bot 30: 440-51

Rubenstein, B. 1974 Effect of pH and auxin on chloride uptake into Avena coleoptile cells. Pl. Physiol 54: 835-9

Ruiter, J.H. 1969 Suspected copper deficiency in radiata pine. Pl. Soil 31: 197-200

Schmid, W.E., H.P. Haag and E. Epstein 1965 Absorption of zinc by excised barley roots. Physiol. Plant 18: 860-9

Schneider, W.C. 1945 Phosphorus compounds in animal tissues. Extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid. J. Biol. Chem 161: 293-303

———— 1957 Determination of nucleic acids in tissues by pentose analysis. p680-4 in Colowick, S.P. and N.O. Kaplan (Eds) Methods in Enzymology. Vol III.

Scott Russell, R. and D.A. Barber 1960 The relationship between salt uptake and the absorption of water by intact plants. Ann. Rev. Pl. Physiol 11: 127-40

Shepherd, K.R. 1965 Growth patterns and growth substances in radiata pine (Pinus radiata D.Don). PhD Thesis, Univ. of Melbourne

Skoog, F. 1940 Relationships between zinc and auxin in the growth of higher plants. Amer. J. Bot 27: 939-51

————, T.C. Broyer and K.A. Grossenbachen 1938 Effects of auxin on rates, periodicity and osmotic relations in exudation. Amer. J. Bot 25: 749-59

Smith, I. (Ed) 1965 Chromatographic and Electrophoretic Techniques. Vol I. William Heinemann, Lond., and Intersci. Publishers Inc., N.Y. 617pp

Smith, M.E. and N.B. Bayliss 1942 The necessity of zinc for Pinus radiata. Pl. Physiol 17: 303-10

Smith, P.F. 1953 Heavy metal accumulation by citrus roots. Bot. Gaz 114: 426-36

————1962 Mineral analysis of plant tissue. Ann. Rev. Pl. Physiol 13: 81-108

———— and A.W. Specht 1953 Heavy metal nutrition and iron chlorosis of citrus seedlings. Pl. Physiol 28: 371-82

Sokal, R.R. and F.J. Rohlf 1969 Biometry. W.H. Freeman & Co., San Francisco 776pp

Spurr, A.R. 1969 A low viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastructure Res 26: 31-43

Spurr, S.H. 1952 Forest Inventory. Ronald Press Co., N.Y. 476pp

Steinberg, R.A. 1956 Metabolism of inorganic nitrogen by plants. p153-8 in McElroy, W.D. and B. Glass (Eds) Inorganic Nitrogen Metabolism. Hopkins Univ. Press, Baltimore, Md.

Steward, F.C. and R.G.S. Bidwell 1966 Storage pools and turnover systems in growing and non-growing cells: experiments with ^{14}C-sucrose, ^{14}C-glutamine and ^{14}C-asparagine. J. Exp. Bot 17: 726-41

Stone, E.I. Jr 1949 Some effects of mycorrhizae on the phosphorus nutrition of monterey pine seedlings. Soil Sci. Soc. Amer. Proc 14: 340-4

Strafford, G.A. 1965 Essentials of Plant Physiology. Heinemann Educ. Books Ltd., Lond. 216pp

Sutcliffe, J.F. 1962 Mineral salts absorption in plants. In International Series of Monographs on Pure and Applied Biology. Vol I. Pergamon Press, N.Y.

Tal, M. and D. Imber 1971 Abnormal stomatal behaviour and hormonal imbalance in Flacca, a wilty mutant of tomato. Pl. Physiol 47: 849-50

Thimann, K.V. 1963 Plant growth substances; past present and future. Ann. Rev. Pl. Physiol 14: 1-18

Thomas, W.A. 1967 Dye and calcium ascent in dogwood trees. Pl. Physiol 42: 1800-2

Thorne, W. 1957 Zinc deficiency and its control. Adv. Agron 9: 31-65

Thurley, K.W. and W.C. Mouel 1974 The etching of thick araldite embedded sections for scanning electron microscopy. J. Microscopy 101: 215-8

Tiffin, L.O. 1966 Iron translocation. I. Plant culture, exudate sampling, iron citrate analysis. Pl. Physiol 41: 510-4

———— 1967 Translocation of manganese, iron, cobalt and zinc in tomato. Pl. Physiol 42: 1427-32

———— and J.C. Brown 1962 Iron chelates in soybean exudate. Science 135: 311-3

Turner, R.G. 1970 The subcellular distribution of zinc and copper within the roots of metal tolerant clones of Agrostis tenuis Sibth. New Phytol 69: 725-31

Vallee, B.L. 1962 Zinc in mineral metabolism. p443 in Comar, C.L. and F. Bonner (Eds) Mineral Metabolism. Vol II, Pt B. Acad. Press, N.Y.

Wacker, W.E.C. 1962 Nucleic acids and metals. III. Changes in nucleic acid, protein and metal content as a consequence of zinc deficiency in Euglena gracilis. Biochem 1: 859-65

———— and B.L. Vallee 1959 Nucleic acids and metals. I. Chromium, manganese, nickel, iron and other metals in ribonucleic acid from diverse biological sources. J. Biol. Chem 234: 3257-62

Wareing, P.F. 1970 Growth and its co-ordination in trees. p1-21 in Luckwill, L.C. and C.V. Cutting (Eds) Physiology of Tree Crops. Acad. Press, Lond., N.Y.

Waygood, E.R., A.Oaks and G.A. McLachlan 1956 The enzymatically catalyzed oxidation of indoleacetic acid. Can. J. Bot 34: 905-26

Welch, R.M. and E. Epstein 1969 The plasmalemma: seat of the type 2 mechanism of salt absorption. Pl. Physiol 44: 301-4

Wieneke, J., O. Biddulph and C.G. Woodbridge 1971 Influence of growth regulating substances on absorption and translocation of calcium in pea and bean. J. Amer. Soc. Hort. Sci 96: 718-20

Wilson, B.F. and E.P. Bachelard 1975 Effects of girdling and defoliation on root activity and survival of Eucalyptus regnans and E. viminalis seedlings. Aust. J. Pl. Physiol 2: 197-206

Wishnick, M., M.D. Lare, M.C. Scrutton and A.S. Mildvar 1969 The presence of tightly bound copper and ribulose diphosphate carboxylase from spinach. J. Biol. Chem 244: 5761-3

Wood, A., L.G. Paleg and R. Southney 1972 Gibberellin and membrane permeability. p37-43 in Carr, D.J. (Ed) Plant Growth Substances 1970. Springer-Verlag, Berlin, Heidlberg, N.Y.

Wood, J.G. and P.M. Sibley 1950 The distribution of zinc in oat plants. Aust. J. Sci. Res B3: 14-27

———— and H.B.S. Womersley 1946 Development and metabolism of copper deficient oat plants. Aust. J. Exp. Biol. and Med. Sci 24: 79-94

Zinkan, C.G., J.K. Jeglum and D.E. Harvey 1974 Oxygen in water culture influences growth and nutrient uptake of jack pine, black spruce and white spruce seedlings. Can. J. Pl. Sci 54: 553-8